Review Article

A Comparison of ToxCast Test Results with In Vivo and Other In Vitro Endpoints for Neuro, Endocrine, and Developmental Toxicities: A Case Study Using Endosulfan and Methidathion

M. Silva,[1] N. Pham,[2] C. Lewis,[1] S. Iyer,[2] E. Kwok,[1] G. Solomon,[3] and L. Zeise[2]

[1] Department of Pesticide Regulation, California Environmental Protection Agency (CalEPA), Sacramento, California
[2] CalEPA's Office of Environmental Health Hazard Assessment (OEHHA), Sacramento, California
[3] Office of the Secretary, CalEPA, Sacramento, California

INTRODUCTION: The U.S. Environmental Protection Agency's (EPA's) Toxicity Forecaster (ToxCast) is a potential tool for chemical prioritization, hazard identification, and risk assessment. We conducted a case study to compare ToxCast data with endpoints from other in vitro and in vivo studies for two data-rich pesticides: endosulfan and methidathion. **METHODS:** ToxCast assays for endocrine disruption, development (zebrafish), and neurotoxicity were qualitatively compared to traditional neurotoxicity, developmental and reproductive toxicity findings. We also used in vitro–in vivo extrapolation to convert half-maximal activity concentrations in active ToxCast assays to rat oral equivalent doses, and quantitatively compared these to the lowest observable effect level (LOEL) from in vivo studies. **RESULTS:** Endosulfan was inactive for $GABA_A R$, unlike in vivo; but active with dopamine transporter assays and was neurotoxic in zebrafish as expected. Methidathion was not active for these endpoints in vivo or in vitro. Acetylcholinesterase inhibition was ToxCast-inactive, although both pesticides are inhibitors in vivo. ToxCast results were generally inactive for endosulfan estrogen receptor agonism and androgen receptor antagonism unlike in vivo. Calculated oral equivalent doses for estrogen receptor and androgen receptor pathways and for zebrafish assays for both compounds were generally consistent with in vivo LOELs. Endosulfan showed neurotoxicity and both pesticides showed developmental effects in the zebrafish assays, although methidathion is not developmentally toxic in vivo. **CONCLUSIONS:** ToxCast's predictions showed concordance on some endpoints and nonconcordance, consisting mainly of false inactives, in several critical endpoints, likely due to a lack of metabolic activation and limitations in assay design. Zebrafish assays were good predictors of developmental toxicity and neurotoxicity for endosulfan. *Birth Defects Res (Part B) 0:1–19, 2015.*   © 2015 Wiley Periodicals, Inc.

Key words: *dopamine/dopamine transporter; endosulfan; endocrine disruptor; estrogen/androgen receptors; high-throughput screening; methidathion; neurotoxicity; ToxCast*

INTRODUCTION AND BACKGROUND

The California Environmental Protection Agency (CalEPA) Department of Pesticide Regulation (CDPR), Office of Environmental Health Hazard Assessment and the Office of the Secretary (OEHHA) are engaged in chemical prioritization and risk assessment on an ongoing basis. Before a risk assessment is performed by CDPR, a pesticide with potential adverse effects must be identified and prioritized within the department (CDPR, 2014). CDPR and OEHHA work together on this process by considering both toxicological and exposure data. Human exposure data along with high-throughput data may someday facilitate chemical prioritization and risk assessment. Also, high-throughput assays may eventually help minimize animal use and the cost of the battery of U.S. Environmental Protection Agency (USEPA) Health Effects Test Guideline studies currently required for pesticide registration (USEPA, 1998) nationally and in California. High-throughput screening (HTS) may also have applications in other programs by providing insights into the potential toxicology of untested

degradation products, consumer product chemicals, and other chemicals that ultimately end up in the environment.

The Toxicity Forecaster (ToxCast) program was launched by the USEPA in 2007 as part of the "Toxicity Testing in the 21st Century (Tox21)" Federal program in collaboration with the National Toxicology Program at the National Institute of Environmental Health Sciences, the National Institutes of Health's National Center for Advancing Translational Sciences, and the Food and Drug Administration (USEPA, 2013). ToxCast was designed to prioritize chemicals based on the results of HTS assays indicating potential disruption of key biological pathways. Chemicals were selected for screening by the USEPA (ToxCast) and the Tox21 collaborators, as well as international programs (OECD) and other stakeholder groups. Currently the multiphase ToxCast program, with over 700 unique assays and 300 signaling pathways, has evaluated numerous chemicals (ffi2000) with established or unknown toxicity, including cosmetics, drugs, pesticides, and environmental contaminants (Tice et al., 2013). The ToxCast data may be used to elucidate biochemical mechanisms as well as common pathways for human disease outcomes. Ultimately a goal of this USEPA program is to use the ToxCast hazard and exposure data predicted by computer modeling to facilitate chemical risk assessments and prioritization.

To test the potential utility of ToxCast for the prioritization and assessment of pesticides, we conducted a case study examining whether the well-established animal bioassay endpoints for two pesticides coincide with results from ToxCast high-throughput assays. Endosulfan (organochlorine) and methidathion (organophosphate) were selected because they have (1) vast in vivo and in vitro databases with well-defined major endpoints that differ substantially from one another; (2) completed risk characterization documents (RCDs) (Lewis, 2007; Silva, 2008); and (3) been tested in numerous ToxCast assays pertaining to endocrine disruption (estrogen receptor (ER), androgen receptor (AR)), developmental toxicity (zebrafish), and neurotoxicity (ffi-aminobutyric acid receptor alpha [$GABA_AR$], acetylcholinesterase [AChE], and dopamine active transporter [DAT]). Our endpoints (neurotoxicity, ER, AR, developmental toxicity) to study ToxCast data were selected because both endosulfan and methidathion are neurotoxins in vivo (through different mechanisms) and show reproductive effects (Lewis, 2007; Silva, 2008; Silva and Gammon, 2009), so we would expect to see active results in ToxCast assays in these domains.

The goals of this case study are to (1) identify areas of concordance and discordance between ToxCast and existing data, including any potential gaps in the ToxCast assays, and (2) attempt translation of oral equivalent doses (OEDs) to evaluate dose concordance for active findings.

METHODS: DATA SOURCES USED

CDPR RCDs

All available traditional in vivo and in vitro data for neurotoxicity and endocrine disruption were analyzed in detail in the endosulfan (CAS: 115-29-7) and methidathion (CAS: 950-37-8) RCDs previously published by CDPR (Lewis, 2007; Silva, 2008) and in Silva and Gam-

mon (2009). Recent articles were also reviewed for this paper. Key neurotoxicity studies reported in the RCDs were selected for inclusion based on their quality and reporting of representative effects.

Pesticide risk assessment involves identification of adverse health effects or toxicity endpoints from studies performed in accordance with Health Effects Test Guidelines (USEPA, 1998) or from the open literature. One approach is to determine the highest pesticide dose at which no significant (biologically and statistically) adverse effect is expected to occur relative to the control group, or the "no-observed-effect-level" (NOEL). This is the treatment dose just below the lowest observed effect level (LOEL), at which a significant increase in adverse effects is observed.

USEPA ToxCast Assays

Results for neurotoxicity and endocrine disruption were obtained from the five ToxCast assay platforms that reported active results for endosulfan and methidathion: ACEA Biosciences, Inc. (ACEA, 6779 Mesa Ridge Rd # 100, San Diego, CA 92121, USA), Attagene (ATG, Attagene, Inc. PO Box 12054 RTP, NC 27709, USA), Novascreen (NVS, 7170 Standard Drive, Hanover Maryland 21076-1334 USA), and Odyssey Thera (OT, 4550 Norris Canyon Road Suite 140 San Ramon, CA 94583, USA); and the NIH Chemical Genomics Center (Tox21, http://ncats.nih.gov/tox21; accessed 5/2015). Table 1 provides detailed information on these assay platforms. Table 1 also includes all available ToxCast assays (numbered) associated with the steps of the estrogen and androgen pathways (for additional information on ER and AR pathways, see Appendix). The assay results for these vendors were obtained from the Interactive Chemical Safety for Sustainability (iCSS) Dashboard (http://actor.epa.gov/dashboard/), the Endocrine Disruptor Screening Program Dashboard (http://actor.epa.gov/edsp21), and the FIFRA SAP Meeting on Integrated Endocrine Activity and Exposure-based Prioritization and Screening (http://www.regulations.gov/; Docket #: EPA-HQ-OPP-2014-0614). Assays reported on the dashboard were performed at multiple concentrations. However, some Novascreen screening assays were performed at one concentration only (25 ffiM for endosulfan and methidathion), and were reported on the iCSS Dashboard in the ToxCast Summary Files (http://www.epa.gov/ncct/toxcast/data.html). Zebrafish results from both the ToxCast program and from academic investigators were obtained from the open literature (Stanley et al., 2009; Padilla et al., 2012; Truong et al., 2014).

Assay Interpretation Methods Used by USEPA

Assays reported on the iCSS Dashboard were run as concentration responses, with one or more replicates pooled and fit to a single curve as described by Filer (2014). Determination of an active (positive) or inactive (negative) response was described on the iCSS Dashboard (Pipeline Overview). Positive values reported by USEPA on the iCSS Dashboard are stated to be "active" and are reported as the concentration at 50% activity (AC_{50}) in micromolars. In assessing whether the ER or AR

Table 1
ToxCast Neurotoxicity, Endocrine Receptor, and Zebrafish Assay Platforms

Vendor	Assay no.	Assay names	Organism/tissue/cell format	Biological process target/target gene (target family)	Reference
Neurotoxicity assays					
NVS	N/A	NVS_LGIC_bGABARaf1[a]	Bovine/brain (cortical membranes)/cell-free	Receptor binding/$GABA_AR1$ (ion channel)	1
	N/A	NVS_LGIC_bGABARa5[a]	Bovine/brain (hippocampal membranes)/cell-free	Receptor binding/$GABA_AR5$ (ion channel)	
	N/A	NVS_LGIC_rGABARa6[a]	Rat/brain (cerebellar membranes)/cell-free	Receptor binding/$GABA_AR$ 6 (ion channel)	
	N/A	NVS_LGIC_bGABAR_Agonist	Bovine/brain (cerebellar membranes)/cell-free	Receptor binding/$GABA_AR$ (ion channel)	
	N/A	NVS_LGIC_GABAR_Nonselective	Rat/brain (whole)/cell-free	Receptor binding/$GABA_AR$ (ion channel)	
	N/A	NVS_ENZ_hAChE	Human/recombinant/cell-free	Regulation of catalytic activity/AChE (esterase)	
	N/A	NVS_ENZ_rAChE	Rat/brain (membranes)/cell-free		
	N/A	NVS_TR_gDAT	Guinea pig/brain (striatal membranes)/cell-free	Receptor binding; SLC6A3 (transporter)	
	N/A	NVS_TR_hDAT	Human/lymphoma cell line (U937)/cell-free		
Estrogen and androgen receptor assays					
ACEA	A12	ACEA_T47D_80hr_Positive	Human/breast/cell-line (T47D)[b]	Cell proliferation/ESR1 (nuclear receptor)	2
ATG	A13	ATG_ERa_TRANS_up[a]	Human/liver/cell line (HepG2)	Regulation of transcription factor activity/ESR1 (nuclear receptor)	3
	B5	ATG_ERE_CIS_up[a]			
		ATG_AR_TRANS_up		Regulation of transcription factor activity/AR (nuclear receptor)	
NVS	A1	NVS_NR_bER	Bovine/uterus (membranes)/cell-free	Receptor binding/ESR1 (nuclear receptor)	4
	A2	NVS_NR_hER	Human/breast cell-line (MCF7)/cell-free		
	A3	NVS_NR_mERa[a]	Mouse uterus/recombinant/cell-free		
	B1	NVS_NR_hAR	Human/prostate cell-line (LnCAP)/cell-free	Receptor binding/AR (nuclear receptor)	
	B2	NVS_NR_cAR	Chimpanzee/recombinant (in sf9/sf21 cells)/cell free		
OT	A4	OT_ER_ERaERa_0480[a]	Human/kidney/cell-line (HEK293T)	Protein stabilization/ESR1 (nuclear receptor)	5
	A5	OT_ER_ERaERa_1440[a]			
	A6	OT_ER_ERaERb_0480[a]			
	A7	OT_ER_ERaERb_1440[a]			
	A8	OT_ER_ERbERb_0480[a]			
	A9	OT_ER_ERbERb_1440[a]			
	A10	OT_ER_EREGFP_0120	Human/cervical/cell-line (HeLa)	Regulation of gene expression/ESR1 (nuclear receptor)	
	A11	OT_ER_EREGFP_0480			
	B3	OT_AR_ARSRC1_0480	Human/kidney/cell-line (HEK293T)	Dimerization through human AR and coupling protein SRC1	
	B4	OT_AR_ARSRC1_0960			
Tox21	A14	Tox21_ERa_BLA_Agonist_ratio[a]	Human/kidney/cell-line (HEK293T)	Regulation of gene expression/ESR1 (nuclear receptor)	6
	A17	Tox21_ERa_BLA_Antagonist_ratio[a]			
	A15	Tox21_ERa_LUC_BG1_Agonist[a]	Human/ovarian/cell-line (BGI)		
	A18	Tox21_ERa_LUC_BG1_Antagonist			
	B6	Tox21_AR_BLA_Agonist_ratio	Human/kidney/cell-line (HEK293T)	Regulation of gene expression/AR (nuclear receptor)	
	B8	Tox21_AR_BLA_Antagonist_ratio			
	B7	Tox21_AR_LUC_MDAKB2_Agonist	Human/breast/cell-line (MDA-kb2)		
	B9	Tox21_AR_LUC_MDAKB2_Antagonist			
Zebrafish					
ZF	N/A	ZF_Total_Score_AC50	Zebrafish (± intact embryo)	Embryonic development	7

References: 1. Novascreen: www.perkinelmer.com; Knudsen et al. (2011); Spies et al. (2013); 2. ACEA biosciences, www.aceabio.com; Rotroff et al. (2013); 3. Attagene, www.attagene.com; Martin et al. (2010); 4. Novascreen, www.perkinelmer.com; 5. Odyssey Thera, www.odysseythera.com; 6. Huang et al. (2011); 7. Padilla et al. (2012); Stanley et al. (2009); Truong et al. (2014). Further ER and AR assay details are available at http://actor.epa.gov/edsp21/. Numbered ToxCast assays, assays formats, and targets described in this table are based on USEPA (2014).
[a] a and b denote e and | forms of the estrogen receptor.
Novascreen assays in italics were tested only at 25 fibM (ToxCast Summary Files available at: http://www.epa.gov/ncct/toxcast/data.html). All other assays were tested at several concentrations as shown on the ICSS Dashboard http://actor.epa.gov/dashboard/.

pathways are active, USEPA considers that a chemical should be active in at least five assays in that particular pathway (Judson et al., 2010).

Activity that may not be specific to the chemical–receptor interaction of interest can also be generated when high chemical concentrations in cell-based receptor-mediated assays initiate a burst of cellular responses indicative of cytotoxicity instead of true chemical–receptor interactions. This area of the dose–response curve, termed the "burst region," is believed to represent an area where true chemical–receptor interactions are obscured due to many factors (activation of multiple cellular pathways, cytotoxicity, and apoptosis) (USEPA, 2014a). USEPA developed methods to calculate specificity (Z-score; USEPA, 2014a) of the chemical–receptor activity, and affinity (Gene Score) of a compound for data interpretation when an assay result (AC_{50}) is near or within the burst region. USEPA interprets a Z-score greater than 3 for a particular assay as reinforcing the likelihood of chemical-receptor specificity. USEPA interprets a Gene Score of greater than 7 as indicating that a compound has strong affinity for a given gene. We obtained the Z-scores from the ToxCast Data Summary Files (http://www.epa.gov/ncct/toxcast/data.html) and calculated the Gene Score.[i]

Another method of data interpretation is an "area under the curve" (AUC) calculation used as a predictor for potency of a given chemical in a specified pathway as detailed in USEPA (2014a). The AUC is an integral across all concentration ranges and all assays (active and inactive) tested for an endpoint or receptor. The modeled data for ER or AR ranges from 0 to 1, in relation to a reference chemical (e.g., 17β-estradiol for ER; USEPA, 2014a). Results that are less than USEPA's cutoff of 0.1 predict that a compound is inactive within the upper limits of concentrations tested for receptors in the defined pathways. Interpretation of AUC data for endosulfan and methidation were provided by USEPA (http://www.regulations.gov/Docket#:EPA-HQ-OPP-2014-0614). AUC values were also compiled to make predictions for ER agonist, antagonist activities, and binding potencies from structure-based models called CERAPP QSAR (Collaborative Integrated Bioactivity and Exposure Ranking Estrogen Receptor Activity Project Quantitative Structure Activity Relationship) (USEPA, 2014a). AUC and CERAPP QSAR information are available at: http://actor.epa.gov/edsp21/.

METHODS: DATA ANALYSIS
Qualitative Concordance Evaluation

The weight of evidence of in vivo and in vitro assays related to neurotoxicity, development, and reproduction, as reported in the RCDs for endosulfan and methidathion,

---

[i] Gene Score: a measure of the potency (log($AC50$)) and specificity (Z-score) for a compound's interaction with a given gene (affinity) calculated as a "Gene Score" as shown below.
Endosulfan Gene Score calculation for ER:
Σ($-\log AC_{50}$s of active ER assays in M units) = 25.74 (logAC_{50}s in http://actor.epa.gov/dashboard/).
Total number of assays for that gene (Table 7).
Σ($-\log AC_{50}$s in M units of active assays)/(total no. of assays for that gene) = 1.43 = x.
Gene Score = 10^x = 26.93.

were compared to the results observed in ToxCast assays related to these endpoints to assess concordance or discordance. We evaluated the utility of the various USEPA criteria for discriminating activity from inactivity in assays and pathways, including the Z-scores and other parameters described above (USEPA, 2014a).

Quantitative Concordance Evaluation

In order to facilitate direct quantitative comparison of active results, we used reverse dosimetry (i.e., in vitro–in vivo extrapolation) to convert any active ToxCast AC_{50} value, including zebrafish; for either chemical into the corresponding OED in rats (Perkins et al., 2013; Wetmore et al., 2013). Using the method and assumptions of Wetmore et al. (2013), metabolic clearance of endosulfan or methidathion by an intact liver in rats was estimated from its metabolic stability data in vitro, number of hepatocytes per gram of liver and liver weight. We scaled the AC_{50} value to an OED of endosulfan or methidathion in rats based on the estimated hepatic metabolic clearance, renal clearance, unbound fraction of parent compound in the plasma, and liver blood flow. Oral equivalent doses[ii] were calculated based on two alternative assumptions in hepatic clearance: restrictive and nonrestrictive (Wetmore et al., 2012). For the former, the hepatic clearance data were scaled by an unbound fraction of the parent compound (F_{ub}), whereas for the latter, the F_{ub} scaling factor was set to 0.99; the F_{ub} values of endosulfan (0.005) and methidathion (0.53) were experimentally derived and reported in the study by Wetmore et al. (2012). The calculated OEDs were used to compare in vivo rat LOEL to the ToxCast AC_{50} (Wetmore et al., 2012; Judson, 2014).

RESULTS
Neurotoxicity

Endosulfan.

In vivo and open literature in vitro findings. Endosulfan acts directly as a noncompetitive $GABA_A R$ antagonist by binding to $GABA_A R\beta$ in the receptor complex and blocking chloride conductance through the ion channels (Kamijima and Casida, 2000; Ratra et al., 2001). This can lead to hyperexcitation of the central nervous system, convulsions, and death. As shown in Table 2, the clinical signs of neurotoxicity observed in rabbits (Nye, 1981), rats (Bury, 1997), mice (Kamijima and Casida, 2000); and Beagle dogs (Brunk, 1989) are symptomatic of $GABA_A R$ inhibition. Mechanistic endpoints of interest are noncompetitive inhibition of $GABA_A R$ at nonselective sites, and inhibition of AChE and DA/DAT (Table 2). Endosulfan decreased dopamine in weanling rat hippocampus contributing to decreased ability to learn and retain a required task compared to controls (Lakshmana and Raju, 1994). Neonatal rats treated with endosulfan had decreased dopamine and increased footshock fighting behavior (Seth et al., 1986). Male offspring (age 3 months) of C57Bl/6j mouse dams gavaged with endosulfan showed a reduction in DAT expression in the central nervous system. Further

---

[ii] Rat OED (mg/kg/d) = ToxCast AC_{50} (μM) × (1 mg/kg/d ÷ RatC$_{SS}$ (μM)).

Table 2
In Vivo Neurotoxic Effects for Endosulfan (Adapted from Silva, 2008)

Species / sex	Exposure info	Effect	NOEL / LOEL (mg / kg / d)	Reference
NZW Rabbit F	Gavage GD 6–28	Symptomatic of GABA$_A$-gated receptor interference: phonation, convulsions / thrashing, hyperactivity, salivation in rabbit does	0.7 / 1.8	Nye (1981)
Wistar Rats M / F	Single gavage	Symptomatic of GABA$_A$-gated receptor interference: tonoclonic convulsions, coarse tremor, uncoordinated gait, increased salivation, stupor, prone position, increased fright reaction, squatting posture, stilted gait, straddled hind limbs, ↓spontaneous activity in females	1.5 / 3.0	Bury (1997)
Swiss-Webster mice	Single i.p.	Measured GABA$_A$R noncompetitive inhibition: myoclonic jerks, head twitching, generalized seizures	No NOEL / 8.0	Kamijima and Casida (2000)
Dog M / F	1 year (diet)	Symptomatic of GABA$_A$-gated receptor interference: violent contractions of the upper abdomen, convulsive movements, extreme sensitivity to noise, frightened reactions to optical stimuli, jerky / tonic muscle contractions (chaps and extremities), impairment of reflex excitability	0.57 / 2.0	Brunk (1989)
Wistar rats M / F	13 week (diet)	↓Plasma (↓41%) and RBC AChE (↓12%) activities in females	2.26 / 4.59	Barnard et al. (1985b)
Wistar rats M / F	13-week neurotoxicity (diet)	↓Plasma AChE (↓50%) activity and convulsions in females	2.88 / 16.6	Sheets et al. (2004)
Wistar Rat weanlings	i.p. 10 or 25 days	↓Dopamine (ffi45%); ↓learning and task retention	6 only dose	Lakshmana and Raju (1994)
ITCR Rat neonate	i.p. PND 1–35	↓Dopamine (27%), ↑Footshock fighting behavior	0.5 / 1.0	Seth et al. (1986)
C57Bl / 6j mice	Dam gavage 14 days premate–PND 21	↓DAT (38%); ↑dopaminergic neurodegeneration; ↓resistance to MPTP challenge at 3 months	1.0 only dose	Wilson et al. (2014)
C57Bl / 6 mice	Pup i.v., PND 5–19; again at 8 months	↓Dopamine (16%) at 8 months after postnatal exposure	1.5 only dose	Jia and Misra (2007)

DAT: dopamine transporter; GD: gestation day; i.p.: intraperitoneal; LOEL: lowest observed effect level; NOEL: no observed effect level; NZW: New Zealand White; PND: postnatal day.

treatment of these male offspring with the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) resulted in additional depletion of DAT (Wilson et al., 2014). C57Bl / 6 mice, exposed to endosulfan as juveniles and treated again as adults, also showed decreased dopamine (Jia and Misra, 2007). Plasma and RBC AChE were decreased in rats (Barnard et al., 1985; Sheets et al., 2004). The tabulated studies in Table 2 are only a few of the many that show neurotoxic effects (Silva, 2008).

ToxCast. Novascreen preliminary screening for bovine-derived GABAR↲1↲, rat-derived GABAR↲1↲, GABA$_A$R agonist, and GABA$_A$R nonselective and human AChE activities were performed with one dose only (25 fflM) for endosulfan and found to be inactive. Therefore a concentration–response assay was not performed. The Novascreen cell-free neurotoxicity assay for bovine GABA$_A$R↲5 was also inactive. A competitive binding assay for DAT performed with guinea pig striatal membranes (gDAT) and the one utilizing human tissue (hDAT) were both active. The calculated OED conversion from the

gDAT (AC$_{50}$ 21.3 fflM) and hDAT (AC$_{50}$ 11.5 fflM) gave OEDs of 0.33 and 0.18 mg / kg / d, respectively. This was threefold and sixfold, respectively, of the LOEL calculated on the basis of the rat in vivo studies (Table 2) (Seth et al., 1986).

Zebrafish. Padilla et al. (2012) did not specify whether or not zebrafish exhibited neurotoxicity as part of the Toxicity Score for endosulfan (mixture of ↲ and | isomers). Stanley et al. (2009) used the more toxic alpha (↲) isomer of endosulfan to test on zebrafish. The most sensitive toxicity endpoint in this assay was an abnormal response the embryo / larvae to touch; suggesting that endosulfan-↲ is developmentally neurotoxic. The NOEL was 0.5 fflM and the LOEL was 1.0 fflM based on abnormal behaviors (prolonged / spastic swimming behavior, disorientation, slower response, and shorter distance swam in response to touch). The calculated OED conversion from the LOEL (1.0 fflM) in zebrafish was 0.038 mg / kg / d. This was 26-fold lower than the 1.0 mg / kg / d, lowest LOEL, from rat in vivo studies (Table 2) from Seth et al. (1986).

Table 3
Characteristic[a] In Vivo Neurotoxic Effects for Methidathion (Lewis, 2007)

Species / sex	Exposure	Effect	NOEL / LOEL (mg / kg / d)	Reference
Crl:CD Rat M / F	Single, gavage	↓ AChE activity in cerebral cortex (M: 74% of controls)	1.0 / 2.2	Chang and Richter (1984)
CrL:CD Rats M / F	90 days, diet	↓ AChE activity in RBCs (M & F: 56–81%), cerebral cortex (M: 74%), striatum (F: 63%) and hippocampus (F: 76%)	No NOEL / 0.6	Chow and Turnier (1995)
Sprague–Dawley rats M / F	104 weeks, diet	Clinical signs, ↓ BW, ↓ food and water consumption, ↓ AChE activity in RBCs (M / F: 78–82%) and whole brain (M / F: 48–49%), ↓ liver weights, skin lesions	1.77	Yau et al. (1986)
Rhesus Monkey M / F	23 months, gavage	↓ AChE in plasma (M / F: 39%) and RBCs (M / F: 60–76%)	1.0	Coulston and Golberg (1971)

[a]The tabulated studies are only a few of the many that show neurotoxic effects (Lewis, 2007).
LOEL: lowest observed effect level; NOEL: no observed effect level.

Methidathion.

In vivo and open literature in vitro findings. Methidathion is an AChE inhibitor (Lewis, 2007). Unlike endosulfan, methidathion requires metabolic activation to exhibit AChE inhibition and neurotoxicity, with the presumed primary toxic metabolite being its oxygen analog (Lewis, 2007). There are currently no in vivo studies indicating methidathion affects the GABA$_A$-gated ionophore complex or DAT (Table 3). The tabulated studies in Table 3 are only a few of the many that show neurotoxic effects in vivo (Lewis, 2007).

ToxCast. Novascreen assay results for AChE, GABA$_A$R, and DAT were reported as inactive in the preliminary single-dose screening at 25 fflM for methidathion, therefore no follow-up multidose testing was done.

Zebrafish. There were no neurotoxicity data available for methidathion.

Assay concordance for neurotoxicity endpoints.
There was a lack of concordance for GABA$_A$R activity for endosulfan (Table 4). In contrast, there was concordance for both gDAT and hDAT and dopamine inhibition studies with in vivo observations. There was no concordance for AChE between in vivo observations and ToxCast assays for endosulfan or methidathion (Table 4).

Endocrine Disruption

Endosulfan.

In vivo and open literature in vitro findings. As shown in Table 5, numerous animal studies and one human epidemiology study have shown adverse effects on reproductive function consistent with disruption of sex hormones. Animal studies have shown uterotropic activity and decreased ovarian weight and functionality in adult females, and decreased ovarian and uterine weights in pups. Male animals have shown related endocrine disrupting effects in gonadal tissues and on post-spermatogenic activities (sperm morphology, motility or function). An epidemiology study reported delayed sexual maturation in boys aged 10 to 19 after prolonged

Table 4
Assay Concordance for Neurotoxicity Endpoints

	Endosulfan	Methidathion
GABA$_A$R To x C d[a]	Inactive	Inactive
GABA-associated in vivo toxicity	Positive	Negative
Concordance	No	Yes
AChE ToxCast[b]	Inactive	Inactive
AChE-associated in vivo toxicity	Positive	Positive[d]
Concordance	No	No
DA / DAT ToxCast[c]	Active	Inactive
DA / DAT-associated in vivo toxicity	Positive	Negative
Concordance	Yes	Yes

[a]GABA$_A$R is bovine derived.
[b]AChE is human derived.
[c]DAT is from guinea pig striatal membranes.
[d]Metabolic activation is necessary for AChE inhibition by methidathion.

exposure to endosulfan used agriculturally. In vitro assays have consistently shown both weak estrogenic and antiandrogenic effects from endosulfan (Table 6).

ER ToxCast assays. Table 7 provides results for ER assays for endosulfan. In the table, a number (1–18) for each assay corresponds to the pathways described in Figures 1 and A1. Endosulfan was active in the ER agonist assays A8, A12, A13, and A15, and was active for ER antagonist assay A18. The active assays involve receptor dimerization, RNA transcription, translation, and cytotoxicity, but not ER receptor binding or ER-induced proliferation.

Only two of the five active assays had a Z-score greater than 3, indicative of specific chemical–receptor interactions by USEPA criteria (assays A12 and A13). The other three assays (assays A8, A15, and A18) were also active on the ToxCast Dashboard but their Z-scores are less than 3, failing the USEPA criterion for specific chemical–receptor interactions and instead potentially reflecting activity due to cell stress. Because only four assays were active for ER agonism while the other was active for antagonism,

Table 5
In Vivo Reproductive and Developmental Effects of Endosulfan (Silva, 2008)

Species/sex	Exposure	Effect	NOEL/LOEL (mg/kg/d)	Reference
Female adults and their offspring				
Uterine tissue from OVX Wistar rat	3 days, s.c.	Adult: ↑uterine ER↓; ↑ER↓ downregulation in uterine subepithelial stroma; uterotropic activity	0.6/6.0	Varayoud et al. (2008)
Hemi-OVX Swiss mice/F	15 day, gavage	Adult: ↓Corpus lutea and healthy follicles, ovary weight; estrus cycles;↑atretic follicles and estrus duration	1.5/3.0	Hiremath and Kaliwal (2002)
Rat pup/F	PND 7–22, s.c.	Pup: ↓E₂; ↓ovarian, oviduct, and uterine weights	No NOEL/4.5	Ahmad et al. (1993)
Sprague–Dawley rat/F	GD 6–14, gavage	Fetal:↓body weight, ↓ %live fetuses and length;↑growth retardation, skeletal anomalies, % resorptions	2.0/6.0	Fung (1980)
Albino rat/F	GD 6–19, gavage	Fetal:↑% skeletal abnormalities	No NOEL/5.0	Gupta et al. (1978)
Crl:COBS(CD)BR rat M/F	2 gen. reproduction, diet (2 litter/gen.)	Pups:↓F1 and F2 mean litter weights	1.2/6.2	Edwards et al. (1984)
Male adults, fetuses, and pups				
Rat pup/M	PND 7–22, s.c.	Pup: ↓Serum T; ↓ reproductive organ weights	No NOEL/4.5	Ahmad et al. (1993)
CD rat/ M/F	13-week diet	Adult: ↑ Epididymal weight	3.85/23.4	Barnard et al. (1985a)
Wistar rat/M	15- or 30-day gavage	Adult: male hormone and aromatase effects[a]	No NOEL/7.5	Singh and Pandey (1989a, 1989c)
Wistar rat/M	7- or 15-day gavage	Adult: ↑ Serum testosterone; ↓testicular testosterone	No NOEL/2.5	Singh and Pandey (1989b)
Wistar rat/M	15- to 30-day gavage	Adult: Male hormone and aromatase effects[b]	5.0/7.5	Singh and Pandey (1990)
Albino rat/M	30-day gavage	Adult:↑Relative testes (to body) weight	2.5/5.0	Dikshith et al. (1984)
ITRC rat/M	15-day gavage	Adult: Effects on male reproductive tract[c]	5.0/10	Gupta and Chandra (1977)
Albino rat/M	30-day gavage	Adult: ↓Spermatogonial cells in metaphase	No NOEL/11.5	Dikshith et al. (1978)
Swiss mice/M	5-day gavage	Adult:↑Chromosomal aberration; abnormal spermatocytes	No NOEL/22	Usha Rani and Reddy (1986)
Druckery rat/M	90-day gavage	↓Sperm, spermatid counts; ↑sperm abnormality	No NOEL/2.5	Sinha et al. (1995, 1997)
Druckrey rat/F	GD 12–21 gavage	↓Sperm, spermatids, ↓Male repro organ weights	No NOEL/1.0	Sinha et al. (2001)
Wistar rat/F	GD 15 through LD 22, gavage	Pups: ↑ Testis weights; ↓ sperm production AND % seminiferous tubules with complete spermatogenesis	>1.5/No LOEL	Dalsenter et al. (2003)
Swiss mice/M	30-day gavage	Adult: ↓ Sperm counts; ↑abnormal sperm	3.0 (only dose)	Khan and Sinha (1996)
Wistar rat/M	6 days/week, 10 weeks, gavage	Adult: ↓Sperm production and count; ↑# abnormal sperm	No NOEL/2.5	Zhu et al. (2002)
Mice M/F	7 days gavage	Adult: ↑Testosterone metabolism and androgen elimination	7.5 (only dose)	Wilson and LeBlanc (1998)
Wistar rat/F	DNT: GD6 to PND 28, diet	Pup: ↓Pup body weight, preputial separation and body weight gain	No NOEL/3.74	Gilmore et al. (2006)
D. melanogaster M	48 h, diet	Adult: ↑Clastogenesis in sperm	No NOEL/50 ppm	Velazquez et al. (1984)
M age 10 to 19 years	Area sprayed 2–3 times/year	Child: ↓SMR (pubic hair, testicular and penis development) and serum T	Unknown	Saiyed et al. (2003)

E₂: 17↓-estradiol; F: female; GD: gestation day; LDT: lowest dose tested; LOEL: lowest observed effect level; M: male; NOEL: no observed effect level; OVX: ovariectomized; PND: postnatal day; s.c.: subcutaneous; SMR: sexual maturity rating.

[a]↓Testosterone, androstenedione, aromatase, and 3↓-& 17↓-hydroxysteroid dehydrogenase; ↑dihydroxytestosterone and androstanediol.

[b]↓Serum testosterone, testicular testosterone, 3-hydroxysteroid-dehydrogenase, and 17–hydroxysteroid-dehydrogenase.

[c]↓Testes weight, seminiferous tubule degeneration, interstitial edema, and spermatogenic elements in tubules.

endosulfan also fails the USEPA criterion that five assays must be active to conclude that a chemical is active in a pathway (Figs. 1 and A1). However, the Gene Score is 26.93, greater than the cutoff value of 7, indicating endosulfan may be specific for the ER gene.

ER in vitro data for endosulfan from registrants and open literature showed activity at concentrations ranging from 1 to 10 ffIM (Table 4), which is similar to the activity levels obtained for active ToxCast ER assays for endosulfan. The AUC for endosulfan in the ER pathway (0.034) predicts inactivity since it falls below the USEPA cutoff of 0.1 (USEPA, 2014a). The CERAPP QSAR model predicted agonist inactivity, very weak antagonist activity and very weak binding potency for endosulfan http://actor.epa.gov/edsp21. In summary, by some criteria, endosulfan would be considered a weak activator of

Table 6
ER or AR In Vitro Assays from Open Literature for Endosulfan (adapted from Silva, 2008)

Cell type	Exposure	Effect	LOEL (ffIM)	Reference
		ER-Related Assays		
Balb / c Mice / F (MMOC); MCF-7 cells	4 days MMOC and 1 day MCF-7	Agonist: ↑end bud and alveolar structures; ↑TERT mRNA MMOC and MCF-7; ↑telomerase activity	MMOC and MCF-1-5	Je et al. (2005)
CHO-K1 cells	1 day	Agonist: ↑ ER⌐ and ER⌐ estrogenic induction (luciferase indicator);	REC$_{20}$ ER⌐ 0.74; RLA 91%; ER⌐ 590; RLA 26%	Kojima et al. (2004)
	8 days	E$_2$ agonist; ↑MCF-7 proliferation (weakly estrogenic)	10	Wade et al. (1997)
MCF-7 cells	6 days	Weak agonist (estrogenic); PE (2.99), RPP (81%); RPE (0.0001%)	10	Soto et al. (1994)
	8 days	E$_2$ agonist; ↑MCF-7 proliferation (weakly estrogenic)	10	Wade et al. (1997)
	6 days	Agonist; ↑cytotoxicity; RPE = 68%, ↑ER transactivation 67% of E2; ↓aromatase	LOEC 1	Andersen et al. (2002)
	6 days	Agonist; PEffi2.3; RPP = 9.54 × 10^{-4}; R PE% = 32%; ↓cells in s-phase	ffi10	Vanparys et al. (2006)
		AR-related assays		
CHO cells	6 days	Antagonist; ↑cytotoxicity; weak antiandrogen	20	Andersen et al. (2002)
CHO-K1 cells	hAR (plasmids)	↓Androgenic activity; ↑AR transcription & DHT conc	69	Kojima et al. (2004)
MDA-kb2 cells	Transfected luciferase gene	↓Androgenic activity	IC$_{50}$ 8.74	Aït-Aïssa et al. (2010)

the ER pathway, whereas other criteria would lead to an interpretation of inactivity.

Table 7 shows the OED (from AC$_{50}$), based on Tox-Cast assay results, that we calculated for endosulfan. The OEDs (range 0.02–0.82 mg / kg / d) are from 3.7- to 310-fold lower than the in vivo LOELs in Table 3 (LOEL range 3.0–6.2 mg / kg / d).

AR Toxcast assays. In Table 8, the assay IDs correspond to the pathways described in Figures 2 and A2. Endosulfan was active in assay B4 for AR agonism, and active in assays B8 and B9 for AR antagonism. The active assays involve receptor dimerization and transcription suppression, but not AR receptor binding, RNA transcription, or protein production. All Z-scores fall below 3 and there were insufficient numbers of active assays for endosulfan to be considered specific for the AR pathway. However, the Gene Score was 16.25, which is greater than the cutoff value of 7, indicating endosulfan may have specificity for the AR gene. AR in vitro data for endosulfan from registrants and the open literature showed antiandrogenic activity at concentrations ranging from 8.74 to 69 ffIM (Table 4), which is similar to the activity levels obtained for active ToxCast AR antagonist assays for endosulfan. The AUC for endosulfan in the AR pathway (0.028) predicts inactivity since it falls below the USEPA cutoff of 0.1 (USEPA, 2014a; http://actor.epa.gov/edsp21. In summary, by most ToxCast interpretation criteria, endosulfan would be considered inactive on the AR pathway; however the elevated Gene Score and activity in a few assays suggests potential activity.

Table 8 shows the OEDs (from AC$_{50}$), based on Tox-Cast assays, that we calculated for endosulfan. The OEDs (range 0.35–0.9 mg / kg / d) were 13- to 32-fold lower than the in vivo LOELs (range 1.0–11.5 mg / kg / d) in Table 3.

Zebrafish. Padilla et al. (2012) showed active results for unspecified malformations in zebrafish (AC$_{50}$ = ffi1 ffIM) after exposure to endosulfan. The Toxicity Score (E_{MAX}) was 40 at peak concentrations (4 ffIM), and is the highest score possible for malformations. In addition, other researchers (Truong et al., 2014) reported a statistically significant increase in malformations from endosulfan treatment at 0.064 ffIM (axis), 0.0064 ffIM (yolk sac edema), and at 64.32 ffIM (pericardial edema) providing a LOEL at 0.0064 ffIM. The calculated OED conversion for zebrafish from the LOEL range of 0.002 to 0.038 mg / kg / d was based on values from Padilla et al. (2012) and Truong et al. (2014), respectively. They were 26- to 500-fold *lower* than the lowest rat reproduction / developmental in vivo LOEL (1.0 mg / kg / d) in Table 5.

Methidathion.

In vivo and open literature studies. Table 9 shows there is evidence that methidathion caused reproductive toxicity, although the effect is not necessarily endocrine mediated. A one-generation two-litter reproductive toxicity study (Salamon, 1986) and a two-generation study (Salamon, 1987) reported a reduced mating index (mating index = #copulations / # estrus cycles) in F$_1$ males. There was evidence of poor maternal care (pups cool to touch, starving,

Table 7
Endosulfan and Methidathion ToxCast ER Assay Results with Dose Response Measures

ID	Assay target[a] (ToxCast assay name[a])	Endosulfan			Methidathion		
		AC_{50} (ffIM)	OED LOEL[b] (mg/kg/d)	Z-score	AC_{50} (ffIM)	OED LOEL[b] (mg/kg/d)	Z-score[c]
	Receptor binding						
NVS nuclear receptor assays							
A1	Bovine ER[j] (NVS_NR_bER)	–	–	–	–	–	–
A2	Human ER[j] (NVS_NR_hER)	–	–	–	–	–	–
A3	Mouse ER[j] (NVS_NR_mERa)	–	–	–	–	–	–
	Dimerization						
OT ER protein complementation assays							
A4	ER[j] -ER[j] dimers (8 hr) (OT_ER_ERaERa_0480)	–	–	–	–	–	–
A5	ER[j] -ER dimers (24 hr) (OT_ER_ERaERa_1440)	–	–	–	–	–	–
A6	ER[j] -ER dimers (8 hr) (OT_ER_ERaERb_0480)	–	–	–	–	–	–
A7	ER -ER dimers (24 hr) (OT_ER_ERaERb_1440)	—	–	–	–	–	–
A8	ER -ER dimers (8 hr) (OT_ER_ERbERb_0480)	10.20	0.17	1.56	–	–	–
A9	ER -ER dimers (24 hr) (OT_ER_ERbERb_1440)	–	–	–	–	–	–
	Protein induction						
OT regulation of gene expression							
A10	ER[j] -ERE (2 hr) (OT_ER_EREGFP_0120)	–	–	–	–	–	–
A11	ER[j] -ERE (8 hr) (OT_ER_EREGFP_0480)	–	–	–	–	–	–
	RNA transcription						
ATG assays							
A12	ER[j] nuclear receptor agonist activity (ATG_ERa_TRANS_up)	1.73	0.03	4.48		–	–
A13	ERE Up/downregulation of endogenous transcription factor activity in human hepatoma (HepG2) cells (ATG_ERE_CIS_up)	1.35	0.02	4.89	28.10	107	5.89
Tox21 ER assays							
A14	Agonist ratio with β-lactamase (ERa_BLA_Agonist_Ratio)	–	–	–	–	–	–
A15	Agonist with BG1 cells LUC reporter (ERa_LUC_BG1_Agonist)	14.50	0.23	0.97	–	–	–
	ER-induced proliferation						
ACEA RT cell growth assays							
A16	T47D cytotoxicity (80 hr) (ACEA_T47D_80hr_Positive)	–	–	–	–	–	–
	Antagonist (transcription suppression)						
Tox21 ER assays							
A17	Antagonist ratio with β-lactamase (ERa_BLA_Antagonist_Ratio)	–	–	–	–	–	–
A18	Antagonist with BG1 cells LUC reporter (ERa_LUC_BG1_Antagonist)	52.30	0.82	−1.14	–	–	–
	Active assay ER-related cytotoxicity						
NA	Cytotoxicity (ACEA_T47D_80hr_Negative)	26.90	0.42	NA	–	–	NA

Adapted from USEPA (2014aa). ER assays used to determine the pathways in Figures 1 and A1 (Appendix) are numbered A1–A18. They are divided according to their assay function as well as the vendor generating the data. LOEL ranges for in vivo effects in females are 3.0 to 6.2 mg/kg/d (Table 3).
[a] Assay name as found on the USEPA iCSS Dashboard (http://actor.epa.gov/dashboard/).
[b] OED: oral equivalent dose (mg/kg) calculation; predicted using 10 ffIM metabolic clearance rate (Wetmore et al., 2012); OED LOEL based on AC_{50} (Wetmore et al., 2012; Judson et al., 2014).
[c] Z-score information is available at: http://www.epa.gov/ncct/toxcast/data.html; ToxCast Summary Files and USEPA (2014aa).

weak, or lethargic) and decreased ovarian weights in the two-generation study F_0 and F_1 females.

Güney et al. (2007) reported ovarian toxicity (Table 9) in female rats from methidathion at 5 mg/kg/d. In vivo findings in the rat reproduction studies were inconclusive as to the mode of action (i.e., direct ER/AR interaction vs. oxidative stress). Ovaries are particularly sensitive to lipid peroxidation from ischemia-perfusion injury related to hydroxyl radicals. Serum malondialdehyde, an indicator of lipid peroxidation, was significantly increased in treated rats. The investigators also note that there is evidence that oxidative stress may be a common mode of action for organophosphate insecticides. It is unclear from these data if the ovarian toxicity was due entirely to the lipid peroxidation or possibly also to endocrine disruption (Güney et al., 2007). Two studies evaluating
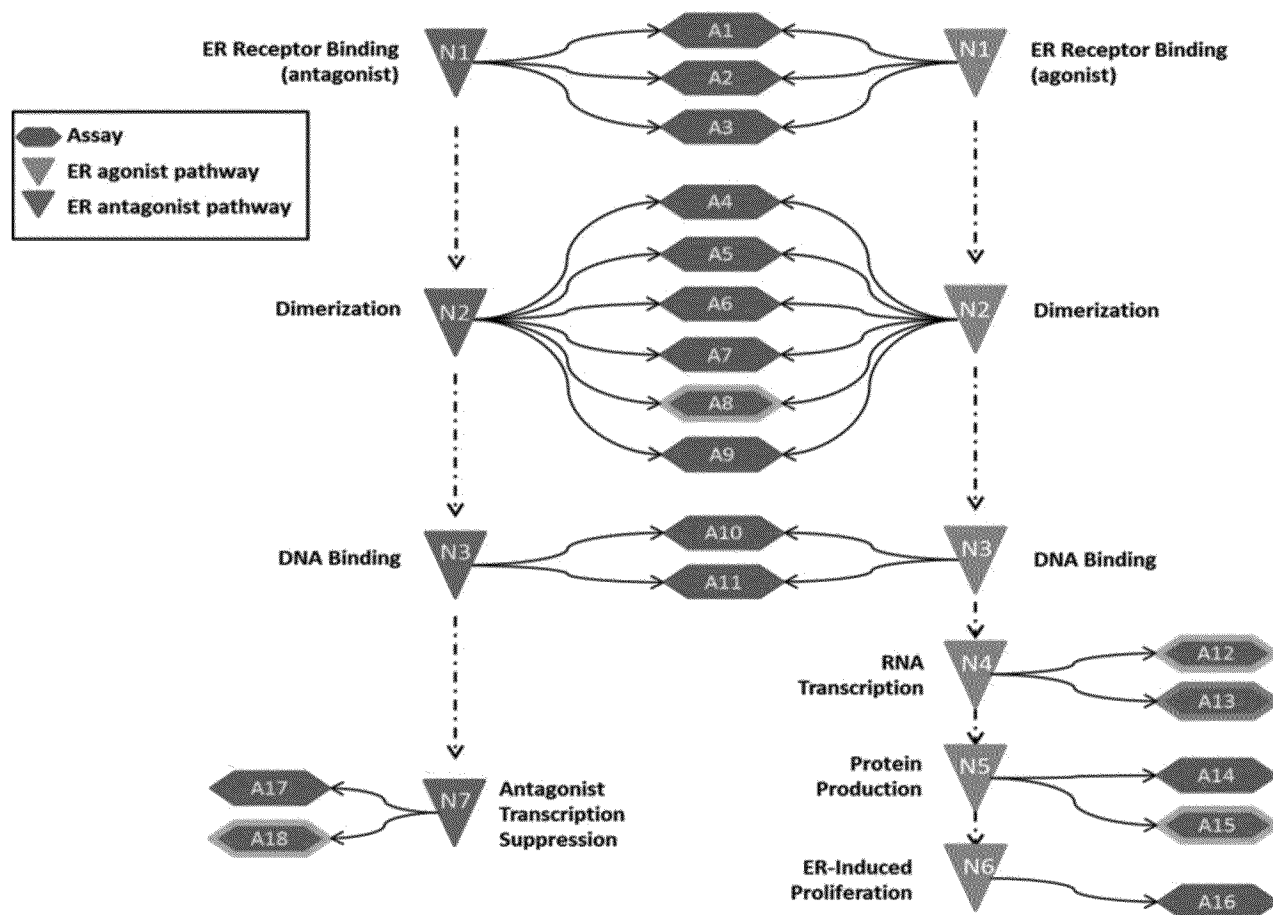
Fig. 1. Graphical representation of the network used in the in vitro analysis of the ER pathway across assays and technology vendors (adapted from USEPA, 2014a). Red and blue colored arrow nodes represent steps where a chemical can directly interact. Dashed arrows represent transfer of information. Green hexagons represents the ER-specific assays tested in ToxCast (see Table 7 for specific description). Highlighted hexagons are assays active for ER with endosulfan (yellow) or for both endosulfan and methidathion (blue).

fetal development did not show developmental toxicity associated with methidathion (Hummel et al., 1987; Mainiero et al., 1987).

ER ToxCast assays. Table 7 provides results for ER assays for methidathion. In the table, a number (1–18) for each assay corresponds to the pathways described in Figures 1 and A1. There was a single active assay in the ER pathway, targeting the estrogen response element (ERE) (A13: ATG_ERE_CIS_up) with an AC_{50} of 28.1 ffIM. The Z-score was 4.68, greater than the USEPA criteria of 3 to define an active result. However, methidathion failed the USEPA criterion that five assays must be active in the ER pathway to conclude that a chemical is active in that pathway (Judson et al., 2010). The Gene Score was 1.7, which is less than the cutoff value of 7, indicating methidathion lacks specificity for the ER pathway. The AUC for methidathion in the ER pathway was zero, confirming that it is virtually inactive with ER (USEPA, 2014a; http://actor.epa.gov/edsp21/). In summary, methidathion activated only the ERE and no other parameters; the elevated Z-score is suggestive of activity, but all other ToxCast interpretation criteria would lead to a conclusion that methidathion is inactive for estrogenicity.

Table 7 shows the calculated OED (from AC_{50}) calculation for methidathion that was based on ToxCast assay results. The OED (107 mg/kg/d) ranges from nine- to 43-fold *higher* than in vivo LOELs (range 3.0–6.2 mg/kg/d) in Table 9.

AR Toxcast assays. Table 8 provides results for AR assays for methidathion. In the table, a number (1–9) for each assay corresponds to the pathways described in Figures 2 and A2. Only one assay (B1) was active for AR and the Z-score (1.4) and the Gene Score (2.6) fell below the cutoff values of 3 and 7, respectively, indicating low potency/specificity for the AR gene. The AUC for methidathion in the AR pathway was zero, predicting that it is virtually inactive with AR (USEPA, 2014a; http://actor.epa.gov/edsp21/).

Table 8 shows the calculated OED (from AC_{50}) for methidathion, based on ToxCast assay results. The OED (84 mg/kg/d) ranges from nine- to 38-fold *higher* than the in vivo LOELs (range 2.2–9.1 mg/kg/d) in Table 3.

Zebrafish. Padilla et al. (2012) reported active results for unspecified malformations for methidathion; the Toxicity Score was 31.5 and the AC_{50} was 45.9 ffIM. After

Table 8
ToxCast AR Assays Performed with Endosulfan and Methidathion

ID	Assay target[a] (ToxCast assay name[b])	Endosulfan AC$_{50}$ (fflM)	Endosulfan OED LOEL[c] (mg/kg/d)	Endosulfan Z-score	Methidathion AC$_{50}$ (fflM)	Methidathion OED LOEL[c] (mg/kg/d)	Methidathion Z-score[d]
	Receptor binding						
NVS nuclear receptor assays							
B1	Human AR (NVS_NR_hAR)	–	–	–	21.90	84.00	6.3
B2	Chimpanzee AR (NVS_NR_cAR)	–	–	–	–	–	–
B3	Rat AR (NVS_NR_rAR)	–	–	–	–	–	–
	Dimerization						
OT AR protein complementation assays							
B4	AR-ARE dimers with agonist activity LUC reporter (24 hr) (OT_AR_ARELUC_AG_1440)	–	–	–	–	–	–
B5	AR-SRC1 dimers (8 hr) (OT_AR_ARSRC1_0480)	–	–	–	–	–	–
B6	AR-SRC1 dimers (16 hr) (OT_AR_ARSRC1_0960)	22.46	0.35	0.25	–	–	–
	RNA transcription						
ATG assays							
B7	AR nuclear receptor agonist activity (ATG_AR_TRANS)	–	–	–	–	–	–
	Protein production						
Tox21 AR assays							
B8	Agonist ratio with ⎮-lactamase (Tox21_AR_BLA_Agonist_ratio)	–	–	–	–	–	–
B9	Agonist activity with MDAKB2–LUC (Tox21_ARa_LUC_MDAKB2_Agonist)	–	–	–	–	–	–
	Antagonist (transcription suppression)						
Tox21 AR assays							
B10	Antagonist ratio with ⎮-lactamase (Tox21_AR_BLA_Antagonist_ratio)	37.44	0.67	−0.58	–	–	–
B11	Antagonist activity with MDAKB2 cells LUC (Tox21_AR_LUC_MDAKB2_Antagonist)	57.19	0.90	−1.29	–	–	–
	Active assays related to cytotoxicity/viability						
NA	Cytotoxicity (Tox21_AR_BLA_Antagonist_viability)	35.90	0.57	NA	–	–	NA

[a] Adapted from USEPA (2014aa). AR assays used to determine the pathways in Figure 2 are numbered B1–B9 and are divided according to their assay function as well as the vendor generating the data.

[b] Assay name as found on the USEPA iCSS Dashboard (http://actor.epa.gov/dashboard/).

[c] OED: oral equivalent dose (mg/kg) calculation; predicted using 10 fflM metabolic clearance rate (Wetmore et al., 2012); OED LOEL based on AC$_{50}$ (Wetmore et al., 2012; Judson et al., 2014)

[d] Z-score information is available at: http://www.epa.gov/ncct/toxcast/data.html; ToxCast Summary Files and USEPA (2014aa).

methidathion treatment other researchers (Truong et al., 2014) showed significantly increased mortality at 0.0064 fflM (LOEL). The OEDs were 0.016 and 174 mg/kg/d based on data from Truong et al. (2014) and Padilla et al. (2012), respectively. The calculated OED conversion for zebrafish from the LOEL range was 79- to 137-fold *higher* than the lowest reproduction in vivo LOEL (2.2 mg/kg/d) in Table 9.

Concordance of ToxCast assays and in vivo studies for endocrine disruption. Endosulfan had weak concordance for in vivo endocrine disruption and ToxCast assays related to the ER. ToxCast data partially support a weak estrogenic mode of action for endosulfan because there were four active assays for agonism and one for antagonism. Although, endosulfan failed two of the USEPA criteria for interpretation of an active result (>five active assays, Z-score >3), the Gene Score for specificity was greater than 7, implying that endosulfan is weakly active in the estrogen pathway according to current criteria. The

AUC analysis, based on ToxCast data, predicts inactive agonist and antagonist activity of endosulfan with the ER (USEPA, 2014a; http://actor.epa.gov/edsp21/). CER-APP QSAR; however predicts very weak antagonistic ER activity for endosulfan. Methidathion had general concordance for equivocal or inactive in vivo estrogenic effects in rat and equivocal or inactive ToxCast assay results for ER. The AUC result of zero and a positive result for only one of three (ERE assay Z-score >3) of the USEPA criteria for an active result would suggest that methidathion does not interact with the ER (AUC: http://www.regulations.gov/ Docket #: EPA-HQ-OPP-2014-0614; http://actor.epa.gov/edsp21/).

There was weak concordance between in vivo endocrine effects in males and ToxCast assays related to AR antagonism by endosulfan. A weak antagonistic mode of action for endosulfan with the AR is shown by previously reported in vivo and in vitro results, but endosulfan failed two of three USEPA criteria for an active interpretation of
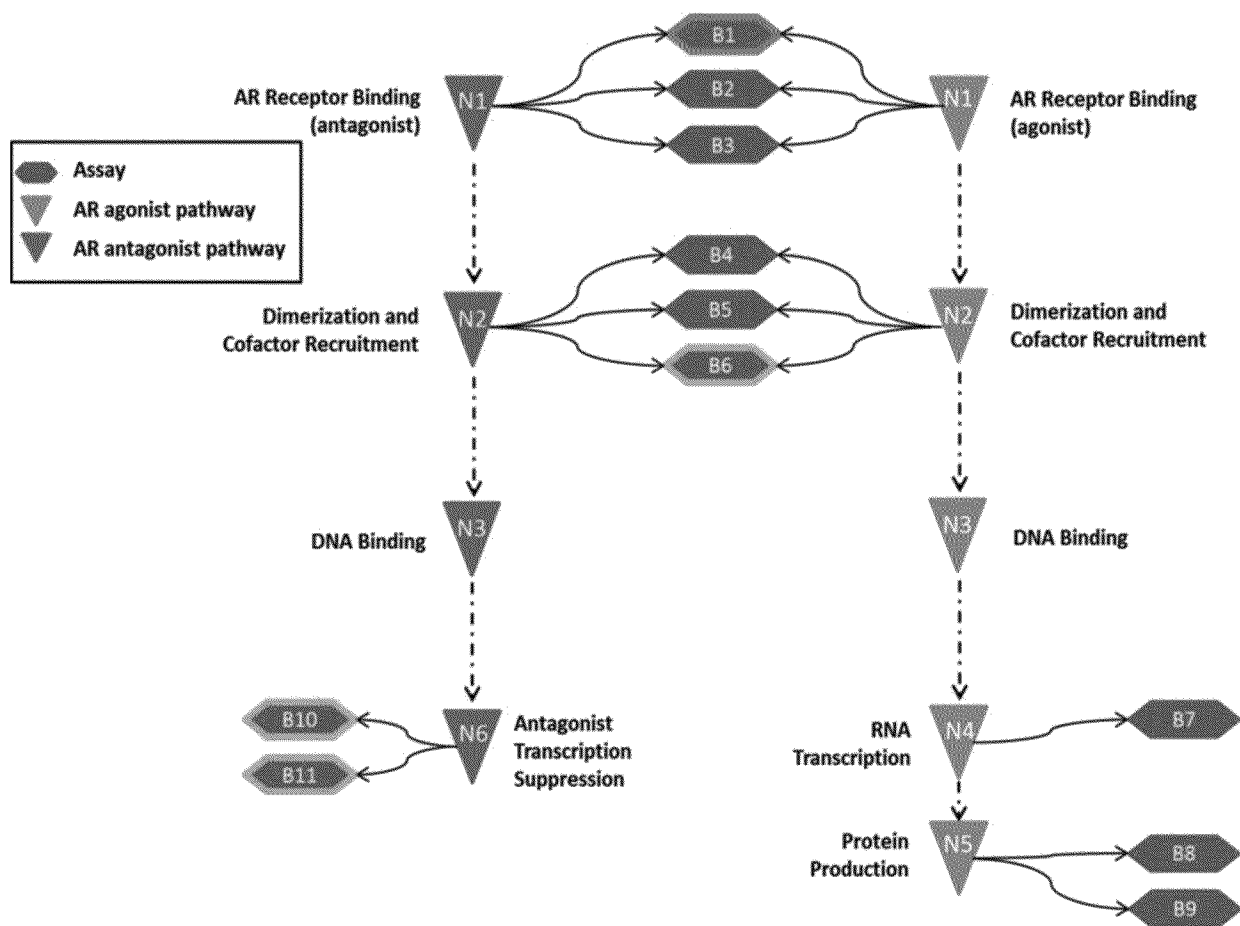
Fig. 2. Graphical representation of the network used in the in vitro analysis of the AR pathway across assays and technology vendors adapted from USEPA (2014aa). Red and blue colored arrow nodes indicate steps with which a chemical can directly interact. Dashed arrows represent transfer of information. Green hexagons represents the AR-specific assays (B1–B9) tested in ToxCast (see Table 8 for specific description). Yellow and blue highlights emphasize assays active for endosulfan and methidathion, respectively.

the AR assays and the AUC also showed antagonist in-activity. The Gene Score, however, reinforces the prediction of weak antagonist activity of endosulfan with the AR. Methidathion had concordance for equivocal in vivo androgenic/antiandrogenic effects in rat and inactive or equivocal ToxCast assay results for AR. Because it was zero, the AUC supported the likelihood of no interaction of methidathion with the AR.

In zebrafish assays endosulfan showed concordance for developmental effects, while methidathion did not. Endosulfan is active for developmental effects in animal models, while methidathion is generally negative. A concordance summary is in Table 10.

DISCUSSION

The large suite of in vitro assays and the zebrafish assay within the ToxCast program has the potential to be a useful tool in the future for screening prioritization and risk assessment. The case studies show what we learned about the current utility of ToxCast assays for our purposes, including certain limitations for prioritization and hazard trait identification for endosulfan and methidathion.

Based on the subset of assays we evaluated here, the ToxCast assay results do not provide much support for in vivo neurotoxicity observations for either compound. The primary targets for the neuroactive pesticides endosulfan and methidathion are known to be $GABA_AR$ and AChE, respectively. The inactivity of endosulfan in the $GABA_AR$ assay may be due to the selection of the binding target in the ToxCast assays. Binding studies using radioligands with high affinity to the receptor blocker site, electro-physiology studies, and molecular modeling have shown that the binding site for $GABA_A$ is located on a different subunit than the binding site for endosulfan and the other insecticidal antagonists, which act as *noncompetitive* blockers of the $GABA_A$-gated chloride channel (Ratra et al., 2001). The lack of metabolic activation in the Novascreen assays does not account for the reported inactivity with endosulfan since the parent compound is neurotoxic (Abalis et al., 1986; Cole and Casida, 1986; Lee et al., 2006); however the fact that the $GABA_AR\|$ with which endosulfan noncompetitively interacts was not the subunit tested may have contributed to the lack of activity. Endosulfan was not active for human AChE in ToxCast assays but it is not known whether it was due to the lack of adequate assay design or another reason. The

Table 9
In Vivo Reproductive Toxicity for Methidathion (Lewis, 2007)

Species / sex	Exposure	Effect	NOEL / LOEL (mg / kg / d)	Reference
		Effects reported in female adults and their offspring		
CR1:CD BR rats / F	2-Gen. repro, diet	↓ Absolute and relative ovary weight (to body)	2.2 / 5.0	Salamon (1987)
Wistar rats / F	5 days / week; 4 weeks, gavage	↓cycles (↓proestrus, estrus and metestrus; ↑ diestrus); ↓ healthy follicles and corpus lutea; ↑atretic follicles with granulosa cells containing abundant lipofuscin pigments; ↑ serum malonaldehyde	No NOEL / 5.0	Güney et al. (2007)
CRI:COBS CD (SD) BR rats: M / F	GD 6–15, gavage	No developmental effects	1.0 / 2.5	Mainiero et al. (1987)
New Zealand White rabbits / F	GD 6–19, gavage	Maternal blood in pan (hemorrhagic discharge); no developmental effects	6.0 / 12	Hummel et al. (1987)
		Effects reported in males		
CD rats: M / F	1-Gen. reprod, 2-litters / gen, diet	↓ Male mating index (#copulations / # estrus cycles)	4.4 / 9.1	Salamon (1986)
CR1:CD BR rats M / F	2-Gen. repro, diet	↓ Male mating index; ↓testes weights	0.4 / 2.2	Salamon (1987)

Table 10
Concordance between ToxCast Assays, Zebrafish, and In Vivo Studies for ER, AR, and Developmental Endpoints

	Endosulfan			Methidathion		
ER ToxCast	Five assays[a]	Gene Score[b]	AUC / CERAPP[c]	Five assays	Gene Score	AUC / CERAPP[c]
	4 / 18	26.93	0.034	1 / 18	1.7	0
	Inactive	Active	Weak antagonist[d]	Inactive	Inactive	Inactive
Estrogenic in vivo and other in vitro		Positive			Negative	
Concordance	No	Yes	Equivocal	Yes	Yes	Yes
AR ToxCast	Five assays	Gene Score	AUC	Five assays	Gene Score	AUC
	3 / 9	16.25	0.028	1 / 9	2.6	0
	Inactive	Active	Inactive	Inactive	Inactive	Inactive
Androgenic in vivo and other in vitro		Active (antiandrogen)			Negative	
Concordance	No	Yes	No	Yes	Yes	Yes
Zebrafish developmental		Strong active			Active	
Developmental toxicity		Positive			Negative	
Concordance		Yes			No	

[a]Pathway is considered active if five assays or more are active.
[b]Pathway is considered active if gene score is greater than 7.
[c]AUC pathway cutoff = 0.1. Adverse male and female reproductive toxicity was observed in the studies without strong indication that estrogenic or androgenic pathways were involved.
[d]Predicted by CERAPP QSAR model.

inactivity of ToxCast assays for AChE with methidathion can be explained by the fact that Novascreen does not include metabolic activation in their in vitro assays. The metabolism of methidathion to its oxygen analog or oxon metabolite is necessary for neurological effects associated with AChE inhibition (Lewis, 2007).

The dopamine pathway assays in ToxCast showed concordance with in vivo endosulfan studies. Wilson et al. (2014) showed that endosulfan treatment decreased DAT levels in male mice treated at critical stages of brain development, leading to greater vulnerability of the dopaminergic neurons to subsequent neurotoxic exposures and potential neurodegenerative diseases. The NVS_TR_DAT assay was active for endosulfan with both guinea pig and human isoforms and LOEL OED estimates were also

in agreement with in vivo rodent study values for the dopamine pathway.

It would be valuable for the ToxCast assay set to include a HTS assay with $GABA_AR$-chloride ion channels intact to positively identify potential for neurotoxicity from ion channel blockers like endosulfan. Intact ion channels would be necessary since the presumed neurotoxicity MOA for compounds like endosufan is noncompetitive $GABA_AR$ antagonism by binding to specific $GABA_AR$ subunits in the receptor complex (Kamijima and Casida, 2000; Ratra et al., 2001). Assays utilizing neural tissues to identify site- and compound-specific activity, perhaps contributing to the understanding of MOAs, would also be useful. Ultimately for AChE inhibitors like methidathion where metabolic activation is critical, whole

cells and metabolic activation might help to identify toxicity from an active metabolite.

In regards to endocrine disruption, the ToxCast assays detected limited activity for endosulfan on the ER and AR pathways, and virtually no activity for methidathion. The endosulfan ranges for the OEDs for ER (0.02–0.82 mg / kg / d) and AR (0.35–0.9 mg / kg / d) in the active assays were comparable to in vivo LOELs. However, despite the plethora of information in open literature relating endosulfan exposure to estrogenic and antiandrogenic effects, including receptor binding, and the reported endocrine disrupting effects in vivo (Table 2), endosulfan was active in only a minimal number of ToxCast assays targeting the ER pathway, none of which included receptor binding assays (Table 7). In addition, endosulfan failed to satisfy some of the ToxCast criteria that categorized a chemical as potent, or active in the ER and AR pathways: too few assays were active, the Z-scores were too low, and the AUC indicated no activity. On the other hand, the Gene Score for endosulfan was positive for both the ER and AR, indicating some specificity for these receptors. A partial explanation for this is that although historically, endosulfan binding to the ER has been associated with weak endocrine effects in vivo (Je et al., 2005; Varayoud et al., 2008), based on the activity observed in the ToxCast assays, endosulfan's estrogenic effects may be due to dimerization and RNA transcription rather than receptor specificity as previously suspected. An additional possible explanation may be that the criteria for defining an active in ToxCast may in this case have excluded true activity; in particular, the criterion of >5 positive assays may need to be reevaluated for defining an estrogenic or antiandrogenic chemical.

In the case of methidathion, the OEDs for the active ToxCast ER and AR assays far exceed the LOELs observed in vivo. While there was observed reduction of ovarian weight and poor maternal care in vivo for methidathion studies, the chemical was weakly active in only two ToxCast assays relating to the endocrine pathways, consistent with the ovarian lipid peroxidation hypothesis, and the idea that the observed in vivo toxicological effects from methidathion exposure may not be directly endocrine mediated.

We found the zebrafish whole organism model to be the best predictor of in vivo effects for the chemicals' hazard traits based on the ToxCast studies of Padilla et al. (2012) as well as the findings of other laboratories (Stanley et al., 2009; Truong et al., 2014). Zebrafish assays predicted neurotoxic effects for endosulfan (consistent with in vivo findings), whereas the other ToxCast assays were mostly inactive. Zebrafish assays were also good predictors of developmental toxicity in that potency agreed strongly with effects observed in vivo with endosulfan being much more potent for all developmental tests performed by Padilla et al. (2012) than methidathion. The greater potency for endosulfan (ZF toxicity score = 40) as a developmental toxicant is consistent with what we would predict based on effects in rodents. The low OEDs for endosulfan in zebrafish emphasized the sensitivity of the zebrafish model for detection of developmental effects compared to other in vivo models. For methidathion, the high OED is somewhat consistent with the negative in vivo findings for developmental toxicity. It should be noted that the zebrafish toxicity scores generated by Padilla et al. (2012) are integrated values summarizing numerous malformations. Exploring specific zebrafish malformations or groups of related malformations could supply further insight on a chemical's effects on developmental cardiotoxicity, neurotoxicity, etc.

From the point of view of a regulatory agency, the use of ToxCast data for prioritization and risk assessment is currently challenging. Based on results posted on the iCSS Dashboard it would not be clear that the main targets for endosulfan and methidathion neurotoxicity are GABA$_A$R and AChE, respectively. Hence they might not be prioritized or assessed appropriately based on the results of these HTS assays.

The analytic approaches to HTS data are undergoing continual refinement at USEPA. The model selection for chemical–response data can affect whether a chemical is classified as "active" or "inactive" in any given assay, as exemplified by comparing some ToxCast chemical activity classifications in USEPA's earlier 2013 data release with the present 2014 data release. For example, we found our case study chemicals to be active in more assays in the present 2014 data release than they were in the earlier 2013 release (e.g., ATG_ERE_up was previously inactive for both endosulfan and methidathion but is now posted as active), due entirely to the data analytic approach used and not to any change in the raw data. Although there are numerous benefits to a transparent process for data availability, the ongoing evolution of the analytical approach poses a challenge for users in data interpretation in chemical hazard identification and prioritization.

An additional issue involves interpretation of the ToxCast data and how without definitive criteria it becomes challenging to determine the pathway activity in the high-throughput screens. Here alone, we have four methods for determining chemical activity: (1) active AC$_{50}$ value, including magnitude of the value to determine assay potency; (2) using Z-scores to distinguish between specific receptor-based chemical activity versus chemical activity resulting in a general cellular response; (3) calculating a Gene Score to synthesize gene-specific chemical data; and (4) using the number of active nodes assayed within a pathway, or AUC calculation, and results of the CERAPP QSAR model as a predictor of potency of a given chemical in a given pathway. One method used to determine chemical activity in high-throughput assays may not lead to the same conclusions as another method, which will affect the conclusions drawn about the chemical's hazard identification. Furthermore, the dynamics of these methods for determining activity may change as new assays are introduced while others are phased out. In the case of endosulfan's estrogenic and antiandrogenic activity, the current methods for determining chemical activity may have resulted in a result that is falsely considered to be inactive. This indicates that the HTS field may need to further develop before results can be reliably used.

There are limitations inherent in comparing endpoint-based in vivo results to pathway-based in vitro results and the overarching goal of correlating assay-level data to higher order biological pathways. The partial lack of concordance between the information in the open literature and in ToxCast could be due to limited understanding of the biological pathways underlying some of the in vivo

observations, or to the complexity of the in vivo pathway interactions compared to the current limited spectrum and range of the ToxCast in vitro assays. Targeting the appropriate molecular targets for evaluating chemical hazard requires selection of high-throughput assays that address a sufficient biologic scope. Limited biological coverage is one of several known challenges within the Tox-Cast program, which are all being considered by USEPA staff (Thomas, 2014; Toxicity Testing in the 21st Century http://www.epa.gov/research/chemicalscience/chemical-toxicitytesting.htm).

In this case study, we examined the potential utility of ToxCast for the prioritization and assessment of pesticides based on data from two well-studied compounds. We identified areas of concordance as well as non-concordance after comparing in vivo data with Tox-Cast assay results. We also noted gaps in the available ToxCast assays leading to apparent false conclusions that indicate inactivity for endosulfan and methidathion. The inactive results that remain unexplained by factors such as metabolic activation include inactivity for endosulfan in GABA$_A$ assays as well as the equivocal findings for endosulfan in the ER and AR assays due to the various criteria for defining an active result. However, when we translated OEDs to evaluate dose concordance for active findings, we observed that the OED calculations for the active ToxCast assays showed surprisingly good agreement for endosulfan—both for endocrine and dopaminergic effects. ER, AR, and zebrafish calculated OEDs for methidathion were also within range of the in vivo LOELs. We also discovered that zebrafish assays were good predictors of developmental toxicity and neurotoxicity with endosulfan.

Ultimately, with improved biological coverage and an expanded knowledge base of chemical activity within ToxCast and other HTS resources, we are optimistic that these in vitro predictive screening programs may account for many of the pathways and toxicological mechanisms needed for understanding chemical toxicity without solely relying on animal models.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The views expressed in this article are those of the authors and do not necessarily reflect the views of policies of the California Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The authors declare no competing financial interest. The opinions and conclusions expressed in this article are those of the authors and do not necessarily represent the views or opinions of the Department of Pesticide Regulation, the Office of Environmental Health Hazard Assessment, or the California Environmental Protection Agency.

APPENDIX

ToxCast Assays Targeting Neurotoxicity Pathways

GABA$_A$ is the major inhibitory neurotransmitter in the central nervous system. The ffi-aminobutyric acid receptor type A (GABA$_A$R) complex comprises the GABA$_A$-gated chloride ion channel within neural membranes (Takeuchi and Takeuchi, 1969; Cole and Casida, 1986; Casida, 1993). The GABA$_A$R activity is modulated at receptor sites, which must include at least one each of the following subunits to function: GABA$_A$R^J 1-6, GABA$_A$R$^|$ -1-3, and GABA$_A$Rffi-1-3 (Ratra et al., 2001). The binding sites are targets for organochlorine pesticides like endosulfan that, upon binding primarily at GABA$_A$R$^|$, noncompetitively block the ion channel (Kamijima and Casida, 2000; Ratra et al., 2001). In brain dopaminergic cells, the DAT membrane protein transports dopamine from neuronal synapses into the cytosol to clear dopamine (DA) from the synapse. In humans, as well as in rodent models, DAT inhibition as well as decreases in dopamine levels can cause hyperactivity and motor and learning deficits during development (Vandenbergh et al., 1992; Kintscher, 2012) and may contribute to neurodegeneration (Jia and Misra, 2007; Wilson et al., 2014). ACh binds to its receptors (nicotinic and muscarinic) in the central and peripheral nervous system to transmit the neural signal. AChE degrades ACh to clear it from synapse (Ellenhorn and Barceloux, 1988). When ACh is not cleared, various neurological signs including ataxia, muscle fasciculations, convulsions, excessive salivation and lacrimation, difficulty in breathing, and death occur (Ellenhorn and Barceloux, 1988; Ecobichon, 2001).

The specific ToxCast assays testing aspects of the above neurotoxic pathways are cell-free assays by Novascreen as described in Table 1.

ToxCast Assays Targeting ER Pathways

The ER is a hormone nuclear receptor critical for development, metabolic homeostasis, and reproduction. Hormone-ligand binding to the ER causes conformational changes that result in subsequent changes in gene expression. The ability of the ER to directly bind to DNA and subsequently regulate the expression of adjacent genes classifies it as a transcription factor (Evans, 1988; Olefsky, 2001). There are two major ER pathways: genomic and nongenomic (A1). The genomic pathway describes the binding of the hormone to the ER located in the cytosol, which triggers many events starting with receptor migration from the cytosol into the nucleus, receptor dimerization, and subsequent receptor binding of the dimer to specific sequences of DNA known as EREs. This complex then recruits other proteins responsible for the transcription of downstream DNA into messenger RNA. The subsequent translation of RNA to protein may result in changes in cellular function. The nongenomic pathway describes the activation of ERs associated with the cell surface membrane by exposure of the cells to the estrogen hormone (Björnström and Sjöberg, 2004; Zivadinovic et al., 2005). ER agonism and antagonism can be tested at numerous points along both of these pathways. Graphical representation of the testing points targeted in the in vitro analysis of the ER pathway in ToxCast is shown in

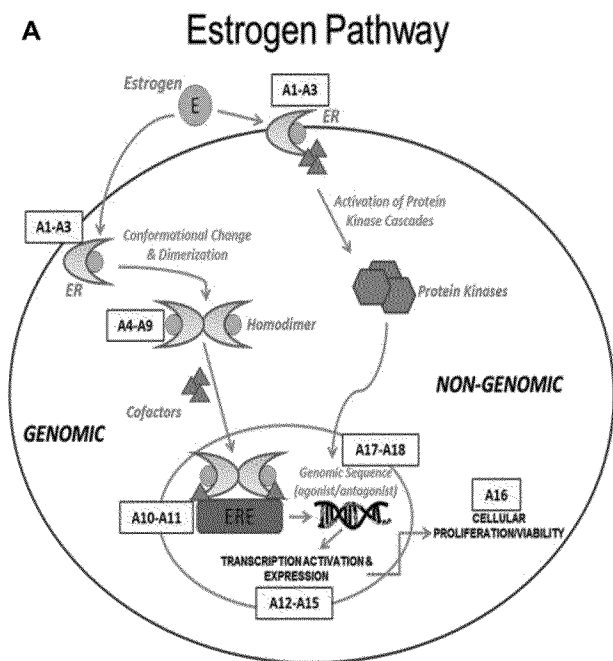**A**   **Estrogen Pathway**

Fig. A1. Genomic and nongenomic estrogen receptor (ER) pathways with ToxCast designated ER assays: A1–A18 (Table 1).

Figure A1 (detailed in USEPA, 2014a). The ToxCast assays, representing several platforms, for the ER pathway are introduced in Table 1.

ToxCast Assays Targeting AR Pathways

The principal steroidal androgens, testosterone, and its metabolite 5^{J}-dihydrotestosterone (DHT), mediate their biological effects predominantly through binding to the AR, an androgen-inducible member of the nuclear receptor superfamily of transcription factors (Lee and Chang, 2003). Androgen binding to AR results in a conformational change in the receptor leading to downstream changes in gene expression. Androgen-regulated genes are critical for the development and maintenance of the male sexual phenotype.

There are two major AR pathways, genomic and nongenomic. The primary mode of action for ARs is genomic, or through direct regulation of gene transcription. Androgen binding to the AR results in a conformational change in the receptor that causes dissociation of heat shock proteins, transportation of the receptor from the cytosol into the cell nucleus, and AR dimerization. The AR dimer then binds to a specific sequence of DNA known as an androgen response element (ARE).

ARs can also have actions that are nongenomic, or independent of their interactions with DNA (Heinlein and Chang, 2002; Fix et al., 2004). Direct androgen binding to cytoplasmic ARs can cause rapid changes in cell function, such as changes in ion transport. Cytoplasmic ARs can also indirectly lead to changes in gene transcription. As referred to above, further descriptions of each assay are in Table 1. The steps of the AR pathway targeted in the ToxCast assays are shown in Figure A2.
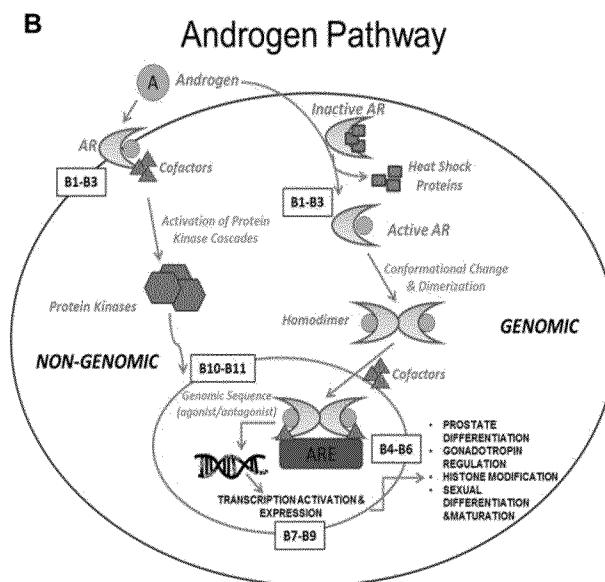
B   **Androgen Pathway**

Fig. A2. Genomic and nongenomic androgen receptor signaling pathways with ToxCast designated AR assays: B1–B9 (Table 1).

Zebrafish Assays

Zebrafish (*Danio rerio*) are known to share many developmental, anatomical, and physiological characteristics with mammals since molecular signaling is known to be conserved across species (Padilla et al., 2012). Zebrafish are especially vulnerable to the toxic effects of chemicals during the first 5 days of development from embryo to adult since changes are the most rapid at this time. If a chemical is developmentally toxic, it would affect molecular pathways or processes in the embryonic zebrafish that may be quickly detected by phenotypic responses. Phenotypic changes can then serve as indicators of affected pathways for target identification (Padilla et al., 2011, 2012; Tanguay et al., 2013; Truong et al., 2014). There are two known approaches to performing high-throughput in vivo assays in zebrafish. Padilla et al. (2012) examine chemical exposure to zebrafish with embryos intact, while Tanguay et al. (2013) remove the chorion of the zebrafish embryo to potentially increase the bioavailability and sensitivity after chemical exposure. The Padilla et al. (2012) method is the one used by the USEPA in ToxCast (Table 1); however the Tanguay et al. (2013) method has also been independently used in their laboratory to test the ToxCast chemicals.

The decision tree used by the USEPA to categorize various morphological endpoints is described by Padilla et al. (2012) and Padilla et al. (2011). If the larva was alive and hatched, then malformations were divided into several categories (e.g., cranial/facial, thorax, abdominal, etc.) and each was scored separately. The summation of scores for all malformation categories is defined as the "Toxicity Score" which ranged from 0 to a maximum value of 40. The minimum cutoff for the Toxicity Score is 6.5 (one standard deviation above the mean of the vehicle control response). Standard sigmoidal curves were fit with the parameters described above (W, AC_{50}, T, B, E_{MAX}). The Toxicity Score and chemical concentration were used to

determine an active result. Chemical potency (AC_{50}) was also determined.

REFERENCES

Abalis IM, Eldefrawi ME, Eldefrawi AT. 1986. Effects of insecticides on GABA induced chloride influx into rat brain microsacs. J Toxicol Environ Health 18:13–23.

Ahmad MM, Maqsood Ahmad M, Sarvat S. 1993. Effects of endosulfan and chlorpyrifos on the reproductive organs and sex hormones of neonatal rats. Pakistan J Zoology 25(1): 11–14.

Aït-Aïssa S, Laskowski S, Laville N, Porcher JM, Brion F. 2010. Anti-androgenic activities of environmental pesticides in the MDA-kb2 reporter cell line. Toxicology in Vitro 24(7): 1979–1985.

Andersen HR, Schmidt IM, Grandjean P, Jensen TK, Budtz-Jorgensen E, Kjaerstad MB, et al. 2008. Impaired reproductive development in sons of women occupationally exposed to pesticides during pregnancy. Environmental health perspectives 116(4): 566–572.

Barnard AV, Jones DR, Powell LAJ, Heywood R, Street AE, Gibson WA, Gopenath C, Majeed SK, Almond R. 1985. 13 Week toxicity study in rats followed by a 4 week withdrawal period (final report). Huntingdon Research Centre, UK; DPR Vol. 182-032 #035803.

Björnström L, Sjöberg M. 2004. Estrogen receptor-dependent activation of AP-1 via non-genomic signalling. Nuclear receptor signaling 2(1): 3.

Brunk R. 1989. Testing for toxicity by repeated oral administration (1 year feeding study) to Beagle dogs. Hoechst Aktiengesellschaft, Germany, Study #: 890188; DPR Vol. 182-065 #074850.

Bury D. 1997. Neurotoxicological screening in the male and female Wistar rat; acute oral toxicity. Hoechst Marion Roussel, Germany; Study #: 960373; Report #: 970149; DPR Vol. 182-106 #162457.

Casida JE. 1993. Insecticide action at the GABA-gated chloride channel: Recognition, progress and prospects. Arch Insect Biochem Physiol 22: 13–23.

CDPR. 2014. Active ingredients prioritized for human health risk assessment initiation. Pesticide Registration and Evaluation Committee Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, CA. Available at www.cdpr.ca.gov. Accessed October 14.

Chang JCF, Richter AG. 1984. Acute neurotoxicity study with methidathion technical in rats. Ciba-Geigy Corp.

Chow E, Turnier JC. 1995. 90-Day subchronic neurotoxicity study with methidathion technical in rats. Ciba-Geigy Corp.

Cole LM, Casida JE. 1986. Polychlorocycloalkane Insecticide Induced Convulsions in Mice in Relation to Disruption of the GABA Regulated Chloride Ionophore. Life sciences 39: 1855–1862.

Coulston F, Golberg, L. 1971. Two-year safety evaluation study in rhesus monkeys with GS 13005. Institute of Experimental Pathology and Toxicology, Ciba-Geigy Corp DPR Vol. 298 014 #935999.

Dalsenter PR, de Araújo SL, da Silva de Assis HC, Andrade AJM. 2003. Pre and postnatal exposure to endosulfan in Wistar rats. Human and Experimental Toxicology 22: 171–175.

Dikshith TSS, Nath G, Datta KK. 1978. Combined cytogenetic effects of endosulfan and metepa in male rats. Indian J Exp Biol 16(1000–1002).

Dikshith TSS, Raizada RB, Srivastava MK, Kaphalia BS. 1984. Response of rats to repeated oral administration of endosulfan. Ind Health 22: 295–304.

Ecobichon DJ. 2001. Toxic Effects of Pesticides. New York: McGraw-Hill.

Edwards JA, Reid YJ, Offu JM, Almond RH, Gibson WA. 1984. Effect of endosulfan technical (Code: HOE 02671 0 I AT209) on reproductive function of multiple generations in the rat. Huntingdon Research Centre, UK. DPR Vol. 182 022 #035789.

Ellenhorn MJ, Barceloux DG. 1988. Pesticides. New York: Elsevier.

Evans RM. 1988. The steroid and thyroid hormone receptor superfamily. Science 240(4854): 889–895.

Filer DL. 2014. The ToxCast analysis pipeline: An R package for processing and modeling chemical screening data. Available at http://epagov/ncct/toxcast/files/MySQL%20Database/Pipeline_Overview.pdf. Accessed January 2015.

Fix C, Jordan C, Cano P, Walker WH. 2004. Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells. Proc Natl Acad Sci USA 101(30): 10919–10924.

Fung WP. 1980. Range finding study with FMC 5462 in pregnant rats. DPR Vol. 182 057 #060605.

Gilmore RG, Sheets LP, Hoss HE. 2006. A Developmental Neurotoxicity Study with Technical Grade Endosulfan in Wistar Rats. Bayer CropScience LP, KS, USA; Report No 201563; 9/26/06 DPR Volume/record #: 182-0122/228573.

Güney M, Demirin H, Oral B, Özgüner M, Bayhan G, Altuntas I. 2007. Ovarian toxicity in rats caused by methidathion and ameliorating effect of vitamins E and C. Hum Exp Toxicol 26:491–498.

Gupta PK, Chandra SV. 1977. Toxicity of endosulfan after repeated oral administration to rats. Bull of Environ Contamin Toxicol 18(3): 378–384.

Gupta PK, Chandra SV, Saxena DK. 1978. Teratogenic and embryotoxic effects of endosulfan in rats. Acta Pharmacol et Toxicol 42(Letter to the editor): 150–152.

Heinlein CA, Chang C. 2002. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. Mol Endocrinol 16(10): 2181–2187.

Hiremath MB, Kaliwal BB. 2002. Effect of endosulfan on ovarian compensatory hypertrophy in hemicastrated albino mice. Repro Toxicol 16:783–790.

Huang R, Xia M, Cho M-H, Sakamuru S, Shinn P, Houck KA, Dix DJ, Judson RS, Witt KL, Kavlock RJ, Tice RR, Austin CP. 2011. Chemical Genomics Profiling of Environmental Chemical Modulation of Human Nuclear Receptors (including Supplemental Material). Environ Health Persp 119: 1142–1148.

Hummel HE, Youreneff MA, Giknis MLA, Arthur AT, Yau ET. 1987. Methidathion—a teratology (segment II) study in rabbits. Ciba Geigy Corp (now Novartis, Basel, Switzerland), Study #: 86; DPR Vol. 298-096 #055140.

Je KH, Kim KN, Nam K-W, Cho M-H, Mar W. 2005. TERT mRNA expression is up-regulated in MCF-7 cells and a mouse mammary culture (MMOC) system by endosulfan treatment. Arch Pharm Res 28(3):351–357.

Jia Z, Misra HP. 2007. Developmental exposure to pesticides zineb and/or endosulfan renders the nigrostriatal dopamine system more susceptible to these environmental chemicals later in life. NeuroTox 24:727–735.

Judson R. 2014. ToxCast: Early trends in the data and data analysis. ToxCast Stakeholder Workshop (January, 2014) US EPA National Center for Computational Toxicology, Office of Research and Development, Washington, DC. Available at http://www.epa.gov/ncct/workshops.html. Accessed October 2014.

Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, Reif DM, Rotroff DM, Shah I, Richard AM, Dix DJ. 2010. In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ Health Persp 118(4):485–492.

Kamijima M, Casida JE. 2000. Regional modification of [3H]ethynylbicycloorthobenzoate binding in mouse brain GABAA receptor by endosulfan, fipronil and avermectin B1a. Toxicol Appl Pharm 163(188-194).

Khan PK, Sinha SP. 1996. Ameliorating effect of vitamin C on murine sperm toxicity induced by three pesticides (endosulfan, phosphamidon and mancozeb). Mutagenesis 11(1): 33–36.

Kintscher U. 2012. Reuptake Inhibitors of Dopamine, Noradrenaline, and Seratonin. Handbook of Experimental Pharmocology 209: 339–347.

Knudsen TB, Houck KA, Sipes NS, Singh AV, Judson RS, Martin MT, Weissman A, Kleinstreuer NC, Mortensen HM, Reif DM, Rabinowitz JR, Setzer RW, Richard AM, Dix DJ, Kavlock RJ. 2011. Activity profiles of 309 ToxCast chemicals evaluated across 292 biochemical targets. Toxicology 282(1–2): 1–15.

Knudsen T, Martin M, Chandler K, Kleinstreuer N, Judson R, Sipes N. 2013. Predictive models and computational toxicology. Methods Mol Biol 947: 343–374.

Kojima H, Katsura E, Takeuchi S, Niiyama K, Kobayashi K. 2004. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese Hamster ovary cells. Environmental Health Perspectives 112(5): 524–531.

Lakshmana MK, Raju TR. 1994. Endosulfan induces small but significant changes in the levels of noradrenaline, dopamine and serotonin in the developing rat brain and deficits in the operant learning performance. Toxicol in Vitro 91(2):139–150.

Lee DK, Chang C. 2003. Molecular communication between androgen receptor and general transcription machinery. J Steroid Biochem Mol Biol 84(1): 41–49.

Lee H-K, Moon J-K, Chang C-H, Choi H, Park H-W, Park B-S, Lee H-S, Hwang E-C, Lee- Y-D, Liu K-H, Kim J-H. 2006. Stereoselective metabolism of endosulfan by human liver microsomes and human cytochrome P450 isoforms. Drug Metab Dispos 37(7):1090–1095.

Lewis CM. 2007. Methidathion (Supracide) risk characterization document (Revision 1). Medical Toxicology Branch, Department of

Pesticide Regulation, California Environmental Protection Agency, Sacramento, CA Health Risk Assessment Volume I. Available at http://www.cdpr.ca.gov/docs/risk/rcd/methidathion_r1.pdf. Accessed October 2014.

Mainiero J, Levy EJ, Infurna RN, Yau ET. 1987. Methidathion technical----a teratology (Segment II) study in rats. Ciba-Geigy Corp (now Novartis, Basel, Switzerland), Study #: 86172 DPR Vol. 298-094 #055139.

Martin MT, Dix DJ, Judson RS, Kavlock RJ, Reif DM, Richard AM, Rotroff DM, Romanov S, Medvedev A, Poltoratskaya N, Gambarian M, Moeser M, Makarov SS, Houck KA. 2010. Impact of Environmental Chemicals on Key Transcription Regulators and Correlation to Toxicity End Points within EPA's ToxCast Program. Chem Res Toxicol 23: 578–590.

Nye D. 1981. Teratology study with FMC 5462 in rabbits. FMC Corp (http://wwwfmccropcom/grower/) Raltech, Study #: FMC A79 370 DPR Vols: 182-027, 057 #s: 035798 and 060607

Olefsky JM. 2001. Nuclear receptor minireview series. J Biol Chem 276(40): 36863–36864.

Padilla S, Hunter DL, Padnos B, Frady S, MacPhail RC. 2011. Assessing locomotor activity in larval zebrafish: Influence of extrinsic and intrinsic variables. Neurotoxicology and Teratology 33(6): 624–630.

Padilla S, Corum D, Padnos B, Hunter DL, Beam A, Houck KA, Sipes N, Kleinstreuer N, Knudsen T, Dix DJ, Reif DM. 2012. Zebrafish developmental screening of the ToxCast Phase I chemical library. Reprod Toxicol 33(2):174–187.

Perkins EJ, Ankley GT, Crofton KM, Garcia-Reyero N, LaLone CA, Johnson MS, Joseph E, Tietge JE, Villeneuve DL. 2013. Current perspectives on the use of alternative species in human health and ecological hazard assessments. Environ Health Persp 121:1002–1010; http://dx.doi.org/1010.1289/ehp.1306638.

Ratra GS, Kamita G, Casida JE. 2001. Role of human GABA^J receptor b3 subunit in insecticide toxicity. Toxicol Appl Pharm 172:233–240.

Rotroff DM, Dix DJ, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, McLaurin KW, Reif DM, Crofton KM, Singh AV, Xia M, Huang R, Judson RS. 2013. Real-Time Growth Kinetics Measuring Hormone Mimicry for ToxCast Chemicals in T-47D Human Ductal Carcinoma Cells. Chemical research in toxicology.

Saiyed H, Dewan A, Bhatnagar V, Shenoy U, Shenoy R, Rajmohan H, et al. 2003. Effect of endosulfan on male reproductive development. Environ Health Perspectives 111(16): 1958–1962.

Salamon C. 1986. Two-generation reproduction study in albino rats. Ciba-Geigy Corp Sponsor (now Novartis, Basel, Switzerland): American Biogenetics Corporation, CA, USA Study #: 450–2125 DPR Vol. 298-097 #055142.

Salamon C. 1987. Methidathion----two-generation reproduction study in rats. Ciba-Geigy Corp Sponsor (now Novartis, Basel, Switzerland) American Biogenetics Corporation, CA, USA Study #: 450–2125 DPR Vol. 298-098 #55143.

Seth PK, Saidi NF, Agrawal AK, Anand M. 1986. Neurotoxicity of endosulfan in young and adult rats. Neurotoxicology 7(2):623–635.

Sheets LP, Gilmore RG, Fickbohm BL. 2004. A subchronic neurotoxicity screening study with technical grade endosulfan in Wistar Rats. Bayer CropScience LP, Toxicology, KS, USA; Lab ID#: 02-N72MJ; Study #: 201069; Report ID#: B004881 DPR Vol.182-0127#234702.

Silva MH. 2008. Endosulfan: Risk Characterization Document. Human Health Assessment Branch, Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, CA. Available at http://www.cdpr.ca.gov/docs/emon/pubs/tac/finaleval/endosulfan.htm. Accessed October 2014.

Silva MH, Gammon D. 2009. An assessment of the developmental, reproductive, and neurotoxicity of endosulfan. Birth Defects Res Part B, Dev Reprod Toxicol 86(1):1–28.

Singh SK, Pandey RS. 1989a. Differential effects of chronic endosulfan exposure to male rats in relation to hepatic drug metabolism and androgen formation. Indian J of Exp Biology 26: 262–267.

Singh SK, Pandey RS. 1989b. Gonadal toxicity of short term chronic endosulfan exposure to male rats. Indian J Exp Biology 27: 341–346.

Singh SK, Pandey RS. 1989c. Toxicity of endosulfan on kidney of male rats in relation to drug metabolizing enzymes and microsomal lipid peroxidation. Indian J of Exp Biology 27: 725–728.

Singh SK, Pandey RS. 1990. Effect of sub-chronic endosulfan exposures on plasma gonadotrophins, testosterone, testicular testosterone and enzymes of androgen biosynthesis in rat. Indian J of Exp Biology 28: 953–956.

Sinha N, Narayan R, Shanker R, Saxena DK. 1995. Endosulfan-induced biochemical changes in the testis of rats. Vet Hum Toxicol 37(6): 547–549.

Sinha N, Narayan R, Saxena DK. 1997. Effect of endosulfan on the testis of growing rats. Bull Environ Contam Toxicol 58(1): 79–86.

Sinha N, Adhikari N, Saxena DK. 2001. Effect of endosulfan during fetal gonadal differentiation on spermatogenesis in rats. Environmental Toxicology and Pharmacology 10: 29–32.

Sipes NS, Martin MT, Kothiya P, Reif DM, Judson R, Richard AM, Houck KA, Dix DJ, Kavlock RJ, Knudsen TB. 2013. Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signaling assays. Chem. Res. Toxicol. 26(6): 878–895.

Soto AM, Chung KL, Sonnenschein C. 1994. The pesticides endosulfan, toxaphene, and dieldrin have estrogenic effects on human estrogen sensitive cells. Environmental health perspectives 102(4): 380–383.

Stanley KA, Curtis LR, Massey Simonich SL, Tanguay RL. 2009. Endosulfan I and endosulfan sulfate disrupts zebrafish embryonic development. Aquat Toxicol 95:355–361.

Takeuchi A, Takeuchi N. 1969. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. J Physiol (Lond) 205(2): 377–391.

Tanguay RL, Truong L, Zaikova T, Hutchison JE. Rapid in vivo assessment of the nano/bio interface. In: Proceedings of the ASME 2013 2nd Global Congress on NanoEngineering for Medicine and Biology, 2013. Boston, MA, USA, February 4–6, 2013. http://proceedings.asmedigitalcollection.asme.org/proceeding.aspx?articleid=1765775 Accessed 10/2014.

Thomas R. 2014. Welcome and overview of data summit objectives. US EPA's chemical safety research: Second ToxCast Data Summit. Available at https://sites.google.com/site/toxcastdatasummit/. Accessed 2014.

Tice RR, Austin CP, Kavlock RJ, Bucher JR. 2013. Improving the human hazard characterization of chemicals: a tox21 update. Environ Health Persp 12(7):756–765.

Toxicity Testing in the 21st Century. Available at http://www.epa.gov/research/chemicalscience/chemical-toxicitytesting.htm.

Truong L, Reif DM, Mary LS, Geier MC, Truong HD, Tanguay RL. 2014. Multidimensional in vivo hazard assessment using zebrafish. Toxicol Sci 137(1):212–233.

Usha Rani MV, Reddy PP. 1986. Cytogenetic effects of aldrin and endosulfan in mice. IRSC Med Sci 14(1125-1126).

USEPA. 1998. Health effect test guidelines. US Environmental Protection Agency. Available at http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPPT-2009-0156-0019. Accessed October 2014.

USEPA. 2013. Toxicity Forecaster (ToxCast^{TM}) advancing the next generation of chemical safety evaluation. Science in Action: Innovative Research for a Sustainable Future Available at http://www2epagov/sites/production/files/2013-12/documents/toxcast-fact-sheetpdf. Accessed April 15.

USEPA. 2014a. Integrated bioactivity and exposure ranking: a computational approach for the prioritization and screening of chemicals in the Endocrine Disruptor Screening Program. US Environmental Protection Agency Endocrine Disruptor Screening Program (EDSP) U.S. EPA Office of Chemical Safety and Pollution Prevention (OCSPP) U.S. EPA Office of Research and Development (ORD); U.S. EPA Office of Water (OW); NIH National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) FIFRA (FIFRA SAP December 2–5, 2014). Available at http://www.regulations.gov/#!documentDetail;D=EPA-HQ-OPP-2014-0614-0003. Accessed 2011/2014.

Vanparys C, Maras M, Lenjou M, Robbens J, Van Bockstaele D, Blust R, et al. 2006. Flow cytometric cell cycle analysis allows for rapid screening of estrogenicity in MCF-7 breast cancer cells. Toxicology in Vitro 20(1238-1248).

Varayoud J, Monje L, Bernhardt T, Munoz-de-Toro M, Luque EH, Ramos GJ. 2008. Endosulfan modulates estrogen-dependent genes like a nonuterotrophic dose of 17b-estradiol. Reprod Toxicol 26:138–145.

Vandenbergh DJ, Persico AM, Hawkins AL, Griffin CA, Li X, Jabs EW, Uhl GR. 1992. Human dopamine transporter gene (DAT1) maps to chromosome 5p15.3 and displays a VNTR. Genomics 14(4): 1104–1106.

Velazquez A, Creus A, Xamena N, Marcos R. 1984. Mutagenicity of the insecticide endosulfan in Drosophila Melanogaster. Mutation Research 136: 115–118.

Wade MG, Desaulniers D, Leingartner K, Foster WG. 1997. Interactions between endosulfan and dieldrin on estrogen-mediated processes in vitro and in vivo. Reproductive Toxicology 11(6): 791–798.

Wetmore BA, Wambaugh JF, Ferguson SS, Sochaski MA, Rotroff DM, Freeman K, Clewell HJ, 3rd, Dix DJ, Andersen ME, Houck KA, Allen B, Judson RS, Singh R, Kavlock RJ, Richard AM, Thomas RS. 2012. Integration of dosimetry, exposure, and high-throughput

screening data in chemical toxicity assessment. Toxicol Sci 125(1):157–174.

Wetmore BA, Wambaugh JF, Ferguson SS, Li L, Clewell III HJ, Judson RS, Freeman K, Bao W, Sochaski MA, Chu T-M, Black MB, Healy E, Allen B, Andersen ME, Wolfinger RD, Thomas RS. 2013. Relative impact of incorporating pharmacokinetics on predicting in vivo hazard and mode of action from high-throughput *in vitro* toxicity assays. Toxicol Sci 132(2):327–346.

Wilson VS, LeBlanc GA. 1998. Endosulfan elevates testosterone biotransformation and clearance in CD-1 mice. Toxicology and applied pharmacology 148: 158–168.

Wilson WW, Shapiro LP, Bradner JM, Caudle WM. 2014. Developmental exposure to the organochlorine insecticide endosulfan damages the nigrostriatal dopamine system in male offspring. Neurotoxicology 44:279–287.

Yau ET, D. McMartin, DN, Zaidi IH, Green JD. 1986. Methidathion – 2-Year oral oncogenicity and toxicity study in albino rats. Ciba-Geigy Corp. DPR Vol. 298 090 #53935.

Zhu X-Q, Zheng Y-F, Zhang Q-W, Jiang H, Huang X-S. 2002. Effects of endosulfan on the spermatogenesis and oxidative damage in rats. Chinese J Pharmacol and Toxicol 14(10): 352–356

Zivadinovic D, Gametchu B, Watson CS. 2005. Membrane estrogen receptor-alpha levels in MCF-7 breast cancer cells predict cAMP and proliferation responses. Breast Cancer Res 7(1): R101–112.